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(54) Title: ISOLATED p27 PROTEIN AND ITS ENCODING NUCLEIC ACID MOLECULES			
(57) Abstract			
<p>The subject invention provides an isolated protein having an apparent molecular weight of about 27 kD and capable of binding to and inhibiting the activation of a cyclin E-Cdk2 complex. The subject invention also provides the nucleic acid encoding the p27 protein, vectors, and host cells for producing the recombinant form of the p27 protein. The subject invention further provides methods for identifying agents capable of modulating the ability of p27 to inhibit the activation of the cyclin E-Cdk2 complex. Finally, the subject invention provides methods for treating subjects diagnosed with hypoproliferative disorders using said agents.</p>			

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Isolated p27 Protein and its Encoding Nucleic Acid Molecules

10 This invention was made with support under Grant No. CA48718 from the National Institutes of Health. Accordingly, the U.S. government has certain rights in the invention.

Background of the Invention

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Throughout this application, various publications are referenced. Full citations for these references may be found at the end of the specification immediately preceding the claims. The disclosures of these publications are
20 hereby incorporated by reference into this application to describe more fully the art to which this invention pertains.

Progression through the cell cycle is marked by a series of
25 irreversible transitions that separate discrete tasks necessary for faithful cell duplication. These transitions are negatively regulated by signals that constrain the cell cycle until specific conditions are fulfilled. Entry into mitosis, for example, is inhibited by incompletely
30 replicated DNA or DNA damage (Weinert and Hartwell, 1988). Another feedback pathway delays the transition from M to G1 if the mitotic spindle is defective (Hoyt et al., 1991; Li & Murray, 1991). These restrictions on cell cycle progression are essential for preserving the fidelity of the
35 genetic information during cell division (Hartwell & Weinert, 1989). The transition from G1 to S phase, on the other hand, coordinates cell proliferation with environmental cues, after which the checks on cell cycle progression tend to be cell autonomous (Hartwell et al.,

1974; Pardee 1974, 1989). Among the extracellular influences that restrict cell cycle progression during G 1 are proteins that inhibit cell proliferation, growth factor or amino acid depletion, and cell-cell contact. Disruption of these
5 signaling pathways uncouples cellular responses from environmental controls and may lead to unrestrained cell proliferation.

Transitions between phases of the cell cycle are catalyzed
10 by a family of cyclin-dependent kinases (Cdks) (Nurse, 1990; Hartwell, 1991). In some organisms the physiological signals controlling the G2 to M transition target a series of steps that activate the mitotic Cdk, Cdc2. Cdc2 activation is positively regulated by phosphorylation on threonine-161
15 (Booher & Beach, 1986; Krek & Nigg, 1991; Gould et al., 1991; Solomon et al., 1990; 1992) and negatively by phosphorylation on tyrosine-15 (Gould & Nurse, 1989). Incomplete DNA replication delays dephosphorylation of tyr-15 (Dasso & Newport, 1990; Smythe & Newport, 1992), and
20 mutations in Cdc2 that convert tyr-15 to a non-phosphorylatable residue are lethal and cause a premature mitosis (Gould & Nurse, 1989). Similarly, either over expression of the tyr-15 phosphatase, Cdc25 (Enoch & Nurse, 1990; Kumagai & Dunphy, 1991), or loss of the tyr-15 kinases
25 (Ludgren et al., 1991) bypass the requirement that DNA replication be completed before mitosis begins. Additional levels of control are probably required to fully explain the block to mitosis caused by ongoing DNA replication (Sorger & Murray, 1992; Heald et al., 1993; Stueland et al., 1993).
30 There is also evidence that cell cycle arrest induced by DNA damage may be related to inactivation of Cdc2 (Rowley et al., 1992; Walworth et al., 1993), but the role of tyrosine phosphorylation in this context has been questioned (Barbet & Carr, 1993).

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There is some evidence, particularly in yeast, that signals inhibiting the G1 to S phase transition block Cdk activation. The mating pheromone alpha factor arrests the *S. cerevisiae* cell cycle in G1 (Reid & Hartwell, 1977), and
5 this correlates with a decrease in CDC28 kinase activity and a decline in the abundance of active complexes containing G1 cyclins and CDC28 (Wittenberg et al., 1990). The FAR1 protein binds to G1 cyclin-CDC28 complexes in cells treated with alpha factor, and this is probably necessary for cell
10 cycle arrest (Chang & Herskowitz, 1990; Peter et al., 1993). Other inhibitors of CDC28 kinase activity have been identified, but their relationship to physiological signals that control cell cycle progression is not known (Mendenhall, 1993; Dunphy & Newport, 1989).

15 Mammalian cells, like yeast, require cyclin-dependent kinases for progression through G1 and entry into S phase (D'Urso et al., 1990; Blow & Nurse, 1990; Furukawa et al., 1990; Fang & Newport, 1991; Pagano et al., 1993; Tsai et al., 1993). Both D and E-type cyclins are rate limiting for
20 the G1 to S transition and both reduce, but do not eliminate, the cell's requirement for mitogenic growth factors (Ohtsubo & Roberts, 1993; Quelle et al., 1993). There is little information, however, concerning the manner
25 by which these cyclins and Cdks are negatively regulated by extracellular signals that inhibit cell proliferation.

It has been studied how two signals that block the cell cycle in G1, cell-cell contact and TGF- β , affect the
30 activity of a G1 cyclin-dependent kinase, Cdk2 (Paris et al., 1990; Elledge & Spotswood, 1991; Koff et al., 1991; Tsai et al., 1991; Elledge et al., 1992; Rosenblatt et al., 1992). The cell cycle of Mv1Lu mink epithelial cells can be arrested in G1 by growth to high density. These contact
35 inhibited cells express both cyclin E and Cdk2, but cyclin

E-associated kinase activity is not present (Koff et al., 1993). Entry into S phase can also be prevented if Mv1Lu cells are released from contact inhibition in the presence of TGF- β , and this correlates with a block to phosphorylation of the Retinoblastoma (Rb) protein (Laiho et al. 1990). Both Cdk2 and Cdk4 have been implicated as Rb kinases (Matsushime et al., 1992; Hinds et al., 1993; Kato et al., 1993; Ewen et al., 1993a; Dowdy et al., 1993), suggesting that TGF- β induced cell cycle arrest may involve inhibition of Cdk2 during G1 (Howe et al., 1991). Consistent with this, cells arrested in late G1 by TGF- β , like contact inhibited cells, express both cyclin E and Cdk2 but do not contain catalytically active cyclin E-Cdk2 complexes (Koff et al., 1993). Cdk4 synthesis is also repressed by TGF- β (Ewen et al., 1993b). The inactivity of Cdk2 together with the absence of Cdk4 may explain the block to Rb phosphorylation in these cells.

It is shown herein that contact inhibited and TGF- β treated cells, but not proliferating cells, contain a titratable excess of a 27 kD protein that binds to the cyclin E-Cdk2 complex and prevents its activation. The inhibitory activity of p27 can be competed by the cyclin D2-Cdk4 complex, suggesting that p27 and cyclin D2-Cdk4 may function within a pathway that transmits growth inhibitory signals to Cdk2.

The subject invention provides an isolated 27 kD protein capable of binding to and inhibiting the activation of a cyclin E-Cdk2 complex. The subject invention further provides related recombinant nucleic acid molecules, host vector systems and methods for making same. Finally, the subject invention provides methods of identifying agents and using agents which act on or mimic p27 function, so as to exploit the regulatory role of p27 in cell proliferation.

Summary of the Invention

The subject invention provides an isolated protein having an
5 apparent molecular weight of about 27 kD as measured by SDS
polyacrylamide gel electrophoresis, and capable of binding
to and inhibiting the activation of a cyclin E-Cdk2 complex.

The subject invention further provides a recombinant nucleic
10 acid molecule which encodes the protein of the subject
invention.

The subject invention further provides a vector comprising
the recombinant nucleic acid molecule of the subject
15 invention.

The subject invention further provides a host vector system
for the production of a protein having an apparent molecular
weight of about 27 kD as measured by SDS polyacrylamide gel
20 electrophoresis, and capable of binding to and inhibiting
the activation of a cyclin E-Cdk2 complex, which comprises
the vector of the subject invention in a suitable host.

The subject invention further provides a method for
25 producing a protein having an apparent molecular weight of
about 27 kD as measured by SDS polyacrylamide gel
electrophoresis, and capable of binding to and inhibiting
the activation of a cyclin E-Cdk2 complex, which comprises
growing the host vector system of the subject invention
30 under conditions permitting the production of the protein
and recovering the protein produced thereby.

The subject invention further provides a method of
determining whether an agent is capable of specifically
35 inhibiting the ability of p27 protein to inhibit the

activation of cyclin E-Cdk2 complex which comprises: (a) contacting suitable amounts of p27 protein, cyclin E, Cdk2 and the agent under suitable conditions; (b) subjecting the p27, cyclin E, Cdk2, and agent so contacted to conditions
5 which would permit the formation of active cyclin E-Cdk2 complex in the absence of p27 protein; (c) quantitatively determining the amount of active cyclin E-Cdk2 complex so formed; and (d) comparing the amount of active cyclin E-Cdk2 complex so formed with the amount of active cyclin E-Cdk2
10 complex formed in the absence of the agent, a greater amount of active cyclin E-Cdk2 complex formed in the presence of the agent than in the absence of the agent indicating that the agent is capable of specifically inhibiting the ability of p27 protein to inhibit the activation of cyclin E-Cdk2
15 complex.

The subject invention further provides a method of determining whether an agent is capable of specifically enhancing the ability of p27 protein to inhibit the
20 activation of cyclin E-Cdk2 complex which comprises: (a) contacting suitable amounts of p27 protein, cyclin E, Cdk2 and the agent under suitable conditions; (b) subjecting the p27, cyclin E, Cdk2, and agent so contacted to conditions which would permit the formation of active cyclin E-Cdk2
25 complex in the absence of p27 protein; (c) quantitatively determining the amount of active cyclin E-Cdk2 complex so formed; and (d) comparing the amount of active cyclin E-Cdk2 complex so formed with the amount of active cyclin E-Cdk2 complex formed in the absence of the agent, a lesser amount
30 of active cyclin E-Cdk2 complex formed in the presence of the agent than in the absence of the agent indicating that the agent is capable of specifically enhancing the ability of p27 protein to inhibit the activation of cyclin E-Cdk2 complex.

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The subject invention further provides a method of treating a subject having a hyperproliferative disorder which comprises administering to the subject a therapeutically effective amount of an agent capable of specifically enhancing the ability of p27 protein to inhibit the activation of cyclin E-Cdk2 complex in the hyperproliferative cells of the subject, so as to thereby treat the subject.

- 10 The subject invention further provides a method of treating a subject having a hypoproliferative disorder which comprises administering to the subject a therapeutically effective amount of an agent capable of specifically inhibiting the ability of p27 protein to inhibit the
15 activation of cyclin E-Cdk2 complex in the hypoproliferative cells of the subject, so as to thereby treat the subject.

The subject invention further provides a method of diagnosing a hyperproliferative disorder in a subject which
20 disorder is associated with the presence of a p27 protein mutation in the cells of the subject, which comprises determining the presence of a p27 protein mutation in the cells of the subject, said mutation being associated with a hyperproliferative disorder, so as to thereby diagnose a
25 hyperproliferative disorder in the subject.

The subject invention further provides a pharmaceutical composition which comprises an effective amount of a recombinant virus capable of infecting a suitable host cell,
30 said recombinant virus comprising the nucleic acid molecule of the subject invention, and a pharmaceutically acceptable carrier.

Finally, this invention provides a method for treating a
35 subject suffering from a hyperproliferative disorder

associated with the presence of a p27 protein mutation in the cells of the subject, which comprises administering to the subject an amount of the pharmaceutical composition of the subject invention effective to treat the subject.

Brief Description of the FiguresFigure 1A

5 Activation of Cdk2 by cyclin E in extracts from
proliferating and growth arrested cells. Cyclin E was added
to extracts from contact inhibited cells (0), and cells
released from contact inhibition for 15 hours in the
presence (0&15) or absence (15) of TGF- β . The 15 hour cells
10 are referred to in the text as "proliferating cells" to
indicate that they are progressing through the cell cycle
and have entered S phase. 0.05 μ l of cyclin E corresponds to
physiological levels of cyclin E in these extracts. The
inset shows the titration of up to 3 times physiological
15 levels of cyclin E. Cyclin E immunoprecipitates were assayed
for histone H1 kinase activity and the results quantitated
using a phosphorimager. Background levels of phosphorylation
observed in the absence of exogenous cyclin E were
subtracted from each sample.

20

Figure 1B

Activation of Cdk2 by cyclin E in extracts from
proliferating and growth arrested cells. Extracts were
prepared from contact inhibited cells (0), and cells
25 released from contact inhibition for 15 hours in the
presence (0&15) or absence (15) of TGF- β . Physiological
amounts of cyclin E were added to various amounts of these
extracts and to mixtures of extracts. Extracts from
proliferating and arrested cells were mixed in the indicated
30 proportions, and the total amount of protein in each mixture
was 75 μ g. The amount of each extract used (μ g) is
indicated. After incubation, cyclin E was immunoprecipitated
and assayed for H1 kinase activity. Results were quantitated
using a phosphorimager. Background levels of phosphorylation
35 observed in the absence of exogenous cyclin E were

subtracted from each sample.

Figure 2A

A Cdk2 inhibitor binds to cyclin E-Cdk2 complexes. The indicated extracts were incubated with a Cdk2-sepharose beads (K), cyclin E-Cdk2 sepharose beads (EK), or blank sepharose beads (0). The Cdk2 beads contained 2-fold more Cdk2 than present in the cell extract. The cyclin E -Cdk2 beads contained approximately 60-fold more cyclin E than was present in the cell extract. After incubation a portion of each supernatant was analyzed by Western blotting to confirm that neither cyclin E nor CDK2 had leached from the matrices. The remainder of the supernatant was assayed for Cdk2 activation by addition of 2X physiological amounts of cyclin E. Cyclin E immunoprecipitates were assayed for H1 kinase activity and the results quantitated using a phsophorimager. Partial depletion of inhibitor by the Cdk2 beads may be attributable to the formation of cyclin-Cdk2 complexes during the incubation with the cell extract.

20

Figure 2B

A Cdk2 inhibitor binds to cyclin E-Cdk2 complexes. Cdk2 was immunoprecipitated from extracts of contact inhibited cells (0), and cells released from contact inhibition for 15 hours in the presence (0&15) or absence (15) of TGF- β . Half of each immunoprecipitate was incubated with cyclin E plus CAK, and the other half underwent mock incubation. Each immunoprecipitate was then assayed for histone H1 kinase activity and the results quantitated using a phosphorimager. In the absence of added CAK, cyclin E had only a very small activating effect on immunoprecipitated Cdk2 (data not shown).

30

Figure 2C

A Cdk2 inhibitor binds to cyclin E-Cdk2 complexes. Effect of

35

kinase inactive Cdk2 on cyclin E activity in extracts from growth arrested cells. Each extract was incubated with 5 fold excess of cyclin E (just at the cyclin E threshold for this lysate), 0.5 fold excess of kinase inactive Cdk2 (Cdk2K), or both. These proportions were chosen based upon empirical determinations of the maximum amount of Cdk2K that could be added without sequestering the majority of the added cyclin E. Cyclin E immunoprecipitates were assayed for H1 kinase activity and the results quantitated using a phosphorimager.

Figure 3A

Activation of Cdc2 by cyclin B. Cyclin B and Cyclin E were added to extracts from cells released from contact inhibition for 15 hours in the presence (0B15) or absence (15) of TGF- β . After addition of cyclins the extracts were divided and immunoprecipitated with either antisera directed to the C-terminus of Cdc2 or the C-terminus of Cdk2. The immunoprecipitates were assayed for H1 kinase activity and the products resolved on a 12% polyacrylamide gel. The reactions labeled "endogenous" contain no added cyclin.

Figure 3B

Activation of Cdc2 by cyclin B. Cyclin B was added to extracts from cells released from contact inhibition for 15 hours in the presence (0B15) or absence (15) of TGF- β . Half of each reaction was supplemented with purified CAK. Cdc2 was immunoprecipitated with antibody directed towards the C-terminus of Cdc2 and assayed for H1 kinase activity. The results were quantitated using a phosphorimager.

Figure 4A

Effect of cyclin D-Cdk4 complexes on cyclin E activity. Extracts were prepared from contact inhibited cells (0), and cells released from contact inhibition for 15 hours in the

presence (0B15) or absence (15) of TGF- β . 0.05 microliters of Sf9 cell lysates containing cyclin D2, Cdk4, cyclin D2-Cdk4 complexes, or complexes containing cyclin D2 bound to catalytically inactive Cdk4 (Cdk4K) were added to these
5 extracts together with physiological amounts of cyclin E. These amounts of cyclin D2 and Cdk4 closely correspond to physiological amounts of these proteins. Cyclin E was immunoprecipitated and assayed for associated histone H1 kinase activity.

10

Figure 4B

Effect of cyclin D-Cdk4 complexes on cyclin E activity. Extracts were prepared from cells released from contact inhibition for 15 hours in the presence (0B15) or absence
15 (15) of TGF- β . 0.05 microliters of Sf9 cell lysates containing the indicated cyclin D-Cdk4 complexes were added to these extracts in the presence of absence of cyclin E. Cyclin E was immunoprecipitated and assayed for associated histone H1 kinase activity.

20

Figure 5A

A 27 kD cyclin E-Cdk2 binding protein. Contact inhibited Mv1Lu cells were released from quiescence by replating at lower density and extracts prepared from 35S-methionine
25 labeled cells at 0 and 15 h. Some cultures were incubated in the presence of 100pM TGF- β for 15 h (0B15). These metabolically labeled extracts were treated as described and bound proteins eluted in sample buffer and analyzed by SDS-PAGE followed by fluorography. Migration of molecular
30 weight markers are shown. 35S-methionine labeled cell extracts were incubated with Cdk2 or cyclin E-Cdk2 complexes and bound proteins eluted in sample buffer. The arrow indicates the migration of a 27kD protein (p27) specifically associated with cyclin E-Cdk2 complexes in extracts from
35 contact inhibited and TGF- β treated cells.

Figure 5B

A 27 kD cyclin E-Cdk2 binding protein. Extracts from metabolically labeled contact inhibited Mv1Lu cells were incubated with varying amounts of Cdk2 or cyclin E-Cdk2 relative to standard conditions and bound proteins analyzed as described below. The presence of p27 is indicated (arrow).

Figure 5C

A 27 kD cyclin E-Cdk2 binding protein. Cyclin D2-Cdk4 complexes prevent binding of p27 to cyclin E-Cdk2. Extracts from contact inhibited cells were preincubated with 4 μ l of baculovirus produced cyclin D2-Cdk4 complex for 30 minutes at 4°C prior to addition of the cyclin E-Cdk2 complex.

Figure 5D

A 27 kD cyclin E-Cdk2 binding protein. Recovery of p27 in Cdk4 immunoprecipitates. Supernatants from 5C were immunoprecipitated with an anti-Cdk4 antiserum and immunoprecipitates were analyzed on 12% SDS-PAGE. The open arrow at 34kD shows the endogenous mink Cdk4 protein, while the closed arrow indicates p27, associated with the cyclin D2-Cdk4 complexes.

Figure 6A

Heat stability of p27 and the Cdk2 inhibitor. p27 binding is heat stable and p27 can be recovered from proliferating cell extracts by heat treatment. Mv1Lu cells were released from contact inhibition for 15 hours with (0 β 15) or without (15) TGF- β 1. Cells were metabolically labeled using 35S-methionine. Prior to incubation with Cdk2 or cyclin E-Cdk2 complexes cell extracts received either no pretreatment or were heated to 100°C for 3 minutes. Note the appearance of p27 (arrow) in heat treated 15h cell extract.

Figure 6B

Heat stability of p27 and the Cdk2 inhibitor. Cdk2 inhibitory activity can be recovered from proliferating cell extracts by heat treatment. Cyclin E associated kinase activity was measured in extracts from asynchronous proliferating cells by immunoprecipitation with antibodies against human cyclin E. Histone H1 was the substrate and results were quantitated using a phosphorimager. Lane 1 - no additions; Lane 2 - the extract was supplemented with 3 times physiological amounts of cyclin E; Lane 3 - as in lane 2 except that heat treated extract from proliferating cells (see methods) was also added to the cell extract.

Figure 6C

Heat stability of p27 and the Cdk2 inhibitor. Cdk2 inhibitory activity was heat stable. Extracts were prepared from contact inhibited cells (0), cells released from contact inhibition for 48 hours in the presence of TGF- β (0 β 48) or asynchronous proliferating cells (Exp). Cyclin E associated kinase activity measured with or without addition of exogenous cyclin E. In the indicated lanes proliferating cell extracts were mixed with an equal amount of extract from nonproliferating cells, that had either been untreated or heated to 100 °C for 5 minutes.

Figure 7A

Inhibition of cyclin E-associated kinase activity by purified p27. Extracts from metabolically labeled contact inhibited Mv1Lu cells were subjected to chromatography on Cdk2 or cyclin E-Cdk2 affinity columns. Bound proteins, eluted in low pH buffer, and were analyzed by SDS-PAGE. p27 present in cyclin E-Cdk2 eluates is shown (arrow).

Figure 7B

Eluates from Cdk2 or cyclin E-Cdk2 columns were precipitated with acetone and renatured (see methods). A portion of each eluate was added to an extract from proliferating cells. Cyclin E was added and cyclin E associated histone H1 kinase activity measured. The cyclin E associated H1 kinase activity was quantitated and plotted as % inhibition relative to extracts receiving no additions.

Figure 7C

- 10 Renatured eluates were incubated with Cdk2 or cyclin E-Cdk2 complexes. p27 (arrow) bound to cyclin E-Cdk2 after renaturation.

Figure 7D

- 15 Eluates from cyclin E-cdk2 columns were fractionated on 12% acrylamide gels. The gels were sliced as shown and proteins were eluted and renatured. A portion of the protein recovered from each gel slice was added together with cyclin E to extracts prepared from proliferating Mv1Lu cells.
- 20 Cyclin E immunoprecipitates were assayed for histone H1 kinase activity.

Figures 8A, 8B, 8C and 8D

- Purification, cyclin E-Cdk2 interaction, and in vitro translation of Kip1.
- 25 A, Heat-treated extracts from quiescent Mv1Lu cells were subjected to cyclin E-Cdk2 affinity chromatography. The eluate was resolved by SDS-PAGE and silver stained. p27^{Kip1} is indicated by an arrow. The broad band is Cdk2-HA, and the 69 kd band is a contaminant present also in blank lanes.
- 30 B, Extracts from metabolically labeled quiescent Mv1Lu cells were precipitated with preimmune rabbit serum (control) or anti-Cdk2 antibody. C. metabolically-labeled p27 obtained by coprecipitation with anti-Cdk2 antibodies as in panel B (in vivo p27) or by

cyclin E-Cdk2 affinity chromatography as in panel A (in vitro p27) was digested with V8 protease and displayed by SDS-PAGE and fluorography. D, In vitro translations containing empty vector (vector) or vector encoding histidine-tagged mouse Kip1 (Kip1) were bound to Ni⁺⁺-NTA-agarose, boiled in sample buffer and resolved by SDS-PAGE.

Figures 9A and 9B

Mammalian Kip1 sequences, and comparison with Cip1/WAF1. A, Amino acid sequences deduced from Kip1 cDNAs from mink (mk), mouse (m) and human (h). Identical amino acids are indicated by dots. The available mink sequence is incomplete at the C-terminus. Peptide sequences obtained from purified Kip1 are underlined. Thick underlining indicates the two sequences that served to design degenerate oligonucleotides for PCR. B, Sequence alignment between human Kip1 and Cip1/WAF1. The putative bipartite nuclear localization signal in both proteins is underlined. A Cdc2 kinase consensus site present in Kip1 is indicated by a thick bar.

Figures 10A, 10B, 10C, 10D and 10E

Cdk inhibition by Kip1 in vitro, and identification of an Cdk inhibitory domain of Kip1. Cell lysates containing baculoviral cyclin E and Cdk2 (A, C) or the indicated cyclin/Cdk combinations, were assayed for histone H1 kinase activity (A, B) and Rb kinase activity (C, D) in the presence of the indicated concentrations of Kip1. Representative gels containing the phosphorylated substrates are shown (A, C). Relative phosphorylation levels were quantitated, and are plotted as the percentage of phosphorylation observed in reactions without Kip1. E, Schematic of the Kip1 protein indicating the regions of highest homology to Cip1/WAF1 (shaded boxes; see also Figure 9B). Bars and numbers indicate the size and location of the

various fragments produced with a C-terminal hexahistidine tag and used in Cdk inhibition assays. The activity of these fragments is presented as a percentage relative to the activity of full length Kip1.

5

Figures 11A and 11B

Kip1 inhibits activation of Cdk2 in vitro. Extracts from exponentially growing A549 cells were incubated with baculovirally expressed histidine-tagged cyclin E alone or together with Kip1. Cyclin E complexes were then retrieved with Ni⁺⁺-NTA-agarose, and assayed for histone H1 kinase activity (A), and by western immunoblotting using anti-Cdk2 antibody (B). Kinase activity was quantitated by Phosphorimager and expressed as arbitrary units. In B, Cdk2* indicates the faster migrating form of Cdk2 that corresponds to Cdk2 phosphorylated at Thr¹⁶⁰ (Gu et al., 1992).

10

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Figures 12A and 12B

Expression pattern of Kip1 in various tissues and cell proliferation states. Kip1 Northern blots using equal amounts of poly(A)⁺ RNA from the indicated human tissues (A) or from Mv1Lu cells in different proliferation states (B). The latter blot was rehybridized with a glyceraldehyde-phosphate dehydrogenase probe.

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25

Detailed Description of the Invention

The subject invention provides an isolated protein having an
5 apparent molecular weight of about 27 kD as measured by SDS
polyacrylamide gel electrophoresis, and capable of binding
to and inhibiting the activation of a cyclin E-Cdk2 complex.

In the subject invention, the SDS polyacrylamide gel
10 electrophoresis used to obtain the 27 kD molecular weight is
performed under reducing conditions.

In one embodiment, the isolated protein of the subject
invention is a mammalian protein. The mammalian protein may
15 be a murine protein. The mammalian protein may also be a
human protein. The mammalian protein may further be a mink
protein. In one embodiment, the mink protein is the mink
protein derived from Mv1Lu cells and having the partial
internal amino acid sequences shown in Table I.

20

Table I

25 Partial Internal Amino Acid Sequences of
Mv1Lu Cell-Derived p27 Protein

30

Thr-Asp-Thr-Ala-Asp-Asn-Gln-Ala-Gly-Leu-Ala-Glu-Gln
Gln-Ala-Val-Pro-Leu-Met-Gly-Pro-Gln-Glu

35

Leu-Pro-Glu-Phe-Tyr-Tyr-Arg-Pro-Pro-Arg-Pro-Pro
Tyr-Glu-Trp-Gln-Glu-Val

40

In the subject invention, the protein having an apparent

molecular weight of about 27 kD as measured by SDS polyacrylamide gel electrophoresis, and capable of binding to and inhibiting the activation of a cyclin E-Cdk2 complex, is referred to synonymously as "p27", "p27 protein",
5 "inhibitor", "p27^{Kip1}" and "Kip1".

As used herein, "isolated" means free of any other proteins. For example, the isolated protein may include nitrocellulose membrane fragments and a buffer.

10

As used herein, "capable of binding to a cyclin E-Cdk2 complex" means capable of binding to a cyclin E-Cdk2 complex but incapable of binding to Cdk2 alone.

15 Inhibition of the activation of a cyclin E-Cdk2 complex may be measured, for example, using assays for (a) the site-specific phosphorylation of the Cdk2 moiety of the cyclin E-Cdk2 complex and (b) histone kinase activity. Such assays are discussed in more detail infra. These assays may be
20 conducted in a kinetic mode (i.e., by measuring the rate of phosphorylation) or as qualitative or quantitative static assays (i.e., measurements made at selected points in time). Those skilled in the art will know that a variety of enzymes and conditions may be used in such assays. In the subject
25 invention, in a kinetic mode assay using equimolar amounts of p27 and cyclin E-Cdk2 complex, p27 inhibits the rate of site-specific phosphorylation of the Cdk2 moiety of the complex (as expressed in moles of Cdk2 moiety phosphorylated per minute) if the rate is inhibited by at least 50%.

30

The isolated 27 kD protein of the subject invention may be obtained, by way of example, by the heat treatment method and by the cyclin E-Cdk2 complex affinity method described infra.

The subject invention further provides a protein comprising a portion having amino acid sequence homology with the portion of p27 protein from amino acid residue +28 to, and including, amino acid residue +88 (as shown in Figure 9B). In one embodiment, the protein has sequence identity with at least one boxed amino acid residue in the portion of p27 protein from amino acid residue +28 to, and including, amino acid residue +88 (as shown in Figure 9B). The protein may be naturally occurring or recombinant. In one embodiment, the degree of homology is 30%. In another embodiment, the degree of homology is 40%. In another embodiment, the degree of homology is 44%. In another embodiment, the degree of homology is 50%. In another embodiment, the degree of homology is 90%.

The subject invention further provides recombinant nucleic acid molecules which encodes the proteins of the subject invention.

20

As used herein, a recombinant nucleic acid molecule is a nucleic acid molecule which does not occur in nature and which is obtained through the use of recombinant technology.

25 In one embodiment, the nucleic acid molecule is a DNA molecule. The DNA molecule may be a cDNA molecule or a cloned genomic DNA molecule. In another embodiment, the nucleic acid molecule is an RNA molecule. The RNA molecule may be an mRNA molecule.

30

The subject invention further provides a vector comprising the recombinant nucleic acid molecule of the subject invention. In one embodiment, the vector is a plasmid. In another embodiment, the vector is a virus.

35

In accordance with the invention, numerous vector systems for expression of the protein of the subject invention may be employed. For example, one class of vectors utilizes DNA elements which are derived from animal viruses such as
5 bovine papilloma virus, polyoma virus, adenovirus, vaccinia virus, baculovirus, retroviruses (RSV, MMTV or MoMLV), Semliki Forest virus or SV40 virus. Additionally, cells which have stably integrated the DNA into their chromosomes may be selected by introducing one or more markers which
10 allow for the selection of transfected host cells. The marker may provide, for example, prototrophy to an auxotrophic host, biocide resistance, (e.g., antibiotics) or resistance to heavy metals such as copper or the like. The selectable marker gene can be either directly linked to the
15 DNA sequences to be expressed, or introduced into the same cell by cotransformation. Additional elements may also be needed for optimal synthesis of mRNA. These elements may include splice signals, as well as transcriptional promoters, enhancers, and termination signals.

20

The subject invention further provides a host vector system for the production of a protein having an apparent molecular weight of about 27 kD as measured by SDS polyacrylamide gel electrophoresis, and capable of binding to and inhibiting
25 the activation of a cyclin E-Cdk2 complex, which comprises the vector of the subject invention in a suitable host.

In one embodiment, the suitable host is a bacterial cell. In another embodiment, the suitable host is an eucaryotic cell.
30 The eucaryotic cell may be an insect cell. Insect cells include, by way of example, sf9 cells.

The subject invention further provides a method for producing a protein having an apparent molecular weight of
35 about 27 kD as measured by SDS polyacrylamide gel

electrophoresis, and capable of binding to and inhibiting the activation of a cyclin E-Cdk2 complex, which comprises growing the host vector system of the subject invention under conditions permitting the production of the protein and recovering the protein produced thereby.

Methods and conditions for growing host vector systems and for recovering the protein so produced are well known to those skilled in the art, and may be varied or optimized depending upon the specific vector and host cell employed. Such recovery methods include, by way of example, gel electrophoresis, ion exchange chromatography, affinity chromatography or combinations thereof.

The subject invention further provides a method of determining whether an agent is capable of specifically inhibiting the ability of p27 protein to inhibit the activation of cyclin E-Cdk2 complex which comprises: (a) contacting suitable amounts of p27 protein, cyclin E, Cdk2 and the agent under suitable conditions; (b) subjecting the p27, cyclin E, Cdk2, and agent so contacted to conditions which would permit the formation of active cyclin E-Cdk2 complex in the absence of p27 protein; (c) quantitatively determining the amount of active cyclin E-Cdk2 complex so formed; and (d) comparing the amount of active cyclin E-Cdk2 complex so formed with the amount of active cyclin E-Cdk2 complex formed in the absence of the agent, a greater amount of active cyclin E-Cdk2 complex formed in the presence of the agent than in the absence of the agent indicating that the agent is capable of specifically inhibiting the ability of p27 protein to inhibit the activation of cyclin E-Cdk2 complex.

As used herein, the term "agent" includes both protein and non-protein moieties. In one embodiment, the agent is a

small molecule. In another embodiment, the agent is a protein. The agent may be derived from a library of low molecular weight compounds or a library of extracts from plants or other organisms.

5

In the subject invention, an agent capable of specifically inhibiting the ability of p27 protein to inhibit the activation of cyclin E-Cdk2 complex interferes with the interaction between p27 protein and cyclin E-Cdk2 complex, but not with the site-specific phosphorylation of the Cdk2 moiety of the cyclin E-Cdk2 complex in the absence of p27 protein.

Cyclin E may be obtained using methods well known to those skilled in the art based on the nucleic acid sequence encoding same as disclosed in Koff, et al. (1991). Cdk2 may be obtained using methods well known to those skilled in the art based on the nucleic acid sequence encoding same as disclosed in Elledge and Spottswood (1991).

20

Amounts of p27 protein, cyclin E, Cdk2 and the agent suitable for the method of the subject invention may be determined by methods well known to those skilled in the art. An example of suitable conditions (i.e., conditions suitable for measuring the effect on p27 function by an agent) under which p27 protein, cyclin E, Cdk2 and the agent are contacted appears infra.

An example of conditions which would permit the formation of active cyclin E-Cdk2 complex in the absence of p27 protein also appears infra.

As used herein, "active cyclin E-Cdk2 complex" means a cyclin E-Cdk2 complex which is capable of specifically phosphorylating a suitable substrate (e.g., histone H1). An

35

example of an active cyclin E-Cdk2 complex is provided infra. The amount of active cyclin E-Cdk2 complex correlates with its measurable activity. Thus, quantitatively determining the amount of active cyclin E-Cdk2 complex may be accomplished by measuring the rate at which a substrate of the active cyclin E-Cdk2 complex is phosphorylated. Such methods well known to those skilled in the art, and include, by way of example, a histone H1 kinase assay.

10

In the method of the subject invention, the cyclin E and Cdk2 proteins may exist as separate proteins, or as a complex, prior to being contacted with the agent.

15 The subject invention further provides a method of determining whether an agent is capable of specifically enhancing the ability of p27 protein to inhibit the activation of cyclin E-Cdk2 complex which comprises: (a) contacting suitable amounts of p27 protein, cyclin E, Cdk2 and the agent under suitable conditions; (b) subjecting the p27 protein, cyclin E, Cdk2, and agent so contacted to conditions which would permit the formation of active cyclin E-Cdk2 complex in the absence of p27 protein; (c) quantitatively determining the amount of active cyclin E-Cdk2 complex so formed; and (d) comparing the amount of active cyclin E-Cdk2 complex so formed with the amount of active cyclin E-Cdk2 complex formed in the absence of the agent, a lesser amount of active cyclin E-Cdk2 complex formed in the presence of the agent than in the absence of the agent indicating that the agent is capable of specifically enhancing the ability of p27 protein to inhibit the activation of cyclin E-Cdk2 complex.

In the subject invention, an agent capable of specifically enhancing the ability of p27 protein to inhibit the

activation of cyclin E-Cdk2 complex affects the interaction between p27 protein and cyclin E-Cdk2 complex, but not with the site-specific phosphorylation of the Cdk2 moiety of the cyclin E-Cdk2 complex in the absence of p27 protein.

5

The subject invention further provides a method of determining whether an agent is capable of mimicking the ability of p27 protein to inhibit the activation of cyclin E-Cdk2 complex which comprises: (a) contacting suitable
10 amounts of cyclin E, Cdk2 and the agent under conditions which would permit the formation of active cyclin E-Cdk2 complex in the absence of the agent; (b) quantitatively determining the amount of active cyclin E-Cdk2 complex so formed; and (c) comparing the amount of active cyclin E-Cdk2
15 complex so formed with the amount of active cyclin E-Cdk2 complex formed in the absence of the agent, a lesser amount of active cyclin E-Cdk2 complex formed in the presence of the agent than in the absence of the agent indicating that the agent is capable of mimicking the ability of p27 protein
20 to inhibit the activation of cyclin E-Cdk2 complex.

The subject invention further provides a method of treating a subject having a hyperproliferative disorder which comprises administering to the subject a therapeutically
25 effective amount of an agent capable of specifically enhancing the ability of p27 protein to inhibit the activation of cyclin E-Cdk2 complex in the hyperproliferative cells of the subject, so as to thereby treat the subject.

30

In the preferred embodiment, the subject is a human.

A hyperproliferative disorder is a disorder wherein cells present in the subject having the disorder proliferate at an
35 abnormally high rate, which abnormally high rate of

proliferation is a cause of the disorder. In one embodiment, the hyperproliferative disorder is selected from the group consisting of cancer and hyperplasia.

- 5 The administering of the agent may be effected or performed using any of the various methods known to those skilled in the art. In one embodiment, the administering comprises administering intravenously. In another embodiment, the administering comprises administering intramuscularly. In
10 yet another embodiment, the administering comprises administering subcutaneously.

The therapeutically effective amount of the agent may be determined by methods well known to those skilled in the
15 art.

The subject invention further provides a pharmaceutical composition comprising a therapeutically effective amount of an agent capable of specifically enhancing the ability of
20 p27 protein to inhibit the activation of cyclin E-Cdk2 complex in the hyperproliferative cells of a subject suffering from a hyperproliferative disorder, and a pharmaceutically acceptable carrier.

- 25 Pharmaceutically acceptable carriers are well known to those skilled in the art and include, but are not limited to, 0.01-0.1M and preferably 0.05M phosphate buffer or 0.8% saline. Additionally, such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions,
30 suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or
35 suspensions, including saline and buffered media.

Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as
5 those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases and the like.

- 10 The subject invention further provides a method of treating a subject having a hyperproliferative disorder which comprises administering to the subject a therapeutically effective amount of an agent capable of mimicking the ability of p27 protein to inhibit the activation of cyclin
15 E-Cdk2 complex in the hyperproliferative cells of the subject, so as to thereby treat the subject.

The subject invention further provides a method of treating a subject having a hypoproliferative disorder which
20 comprises administering to the subject a therapeutically effective amount of an agent capable of specifically inhibiting the ability of p27 protein to inhibit the activation of cyclin E-Cdk2 complex in the hypoproliferative cells of the subject, so as to thereby treat the subject.

25

In the preferred embodiment, the subject is a human.

A hypoproliferative disorder is a disorder wherein cells present in the subject having the disorder proliferate at an
30 abnormally low rate, which abnormally low rate of proliferation is a cause of the disorder. In one embodiment, the hypoproliferative disorder is an ulcer. Examples of hypoproliferative cells are terminally differentiated cells in normal tissue and organs which, with
35 the exception of the liver and bone marrow, normally lack

the ability to regenerate following traumatic injury. Thus, the method of the subject invention, and agents identified thereby, have use in stimulating tissue and organ repair in subjects in need thereof, as well as in establishing tissue
5 cultures of cells from a variety of different tissues.

The therapeutically effective amount of the agent may be determined by methods well known to those skilled in the art.

10

The subject invention further provides a pharmaceutical composition comprising a therapeutically effective amount of an agent capable of specifically inhibiting the ability of p27 protein to inhibit the activation of cyclin E-Cdk2
15 complex in the hypoproliferative cells of a subject suffering from a hypoproliferative disorder, and a pharmaceutically acceptable carrier.

The subject invention further provides a method for
20 obtaining partially purified polyclonal antibodies capable of specifically binding to p27 protein which method comprises (a) immunizing a subject with p27 protein, (b) recovering from the immunized subject serum comprising antibodies capable of specifically binding to p27 protein,
25 and (c) partially purifying the antibodies present in the serum, thereby obtaining partially purified polyclonal antibodies capable of specifically binding to p27 protein.

As used herein, partially purified antibodies means a
30 composition which comprises antibodies which specifically bind to p27 protein, and consists of fewer protein impurities than does the serum from which the antibodies are derived. A protein impurity means a protein other than the antibodies specific for p27 protein. For example, the
35 partially purified antibodies might be an IgG preparation.

Methods of recovering serum from a subject are well known to those skilled in the art. Methods of partially purifying antibodies are also well known to those skilled in the art, and include, by way of example, filtration, ion exchange chromatography, and precipitation.

The subject invention further provides the partially purified antibodies produced by the method of the subject invention.

The subject invention further provides a method for obtaining a purified monoclonal antibody capable of specifically binding to p27 protein which method comprises (a) immunizing a subject with p27 protein, (b) recovering from the immunized subject a B cell-containing cell sample, (c) contacting the B cell-containing cell sample so recovered with myeloma cells under conditions permitting fusion of the myeloma cells with the B cells therein so as to form hybridoma cells, (d) isolating from the resulting sample a hybridoma cell capable of producing a monoclonal antibody capable of specifically binding to p27 protein, (e) growing the hybridoma cell so isolated under conditions permitting the production of the monoclonal antibody, and (f) recovering the monoclonal antibody so produced, thereby obtaining a purified monoclonal antibody capable of specifically binding to p27 protein. Methods of making hybridomas and monoclonal antibodies are well known to those skilled in the art.

30

The subject invention further provides the hybridoma cell produced in step (d) of the method of the subject invention.

The subject invention further provides the purified monoclonal antibody produced by the method of the subject

35

invention.

As used herein, a "purified monoclonal antibody" means the monoclonal antibody free of any other antibodies.

5

The subject invention further provides an antibody capable of specifically binding to p27 protein, said antibody being labeled with a detectable marker.

10 The labeled antibody may be a polyclonal or monoclonal antibody. In one embodiment, the labeled antibody is a purified labeled antibody. The term "antibody" includes, by way of example, both naturally occurring and non-naturally occurring antibodies. Specifically, the term "antibody"
15 includes polyclonal and monoclonal antibodies, and fragments thereof. Furthermore, the term "antibody" includes chimeric antibodies and wholly synthetic antibodies, and fragments thereof. The detectable marker may be, for example, radioactive or fluorescent. Methods of labeling antibodies
20 are well known in the art.

The subject invention further provides a method for quantitatively determining the amount of p27 protein in a sample which comprises contacting the sample with the
25 antibody of the subject invention under conditions permitting the antibody to form a complex with p27 protein present in the sample, quantitatively determining the amount of complex so formed, and comparing the amount so determined with a known standard, so as to thereby quantitatively
30 determine the amount of p27 protein in the sample.

The sample may be, for example, a cell sample, tissue sample, or protein-containing fluid sample. Conditions
35 permitting an antibody to form a complex with its antigen and methods of detecting the presence of complex so formed

are well known in the art.

The amount of p27 protein present in a sample as determined need not be an absolute number, in the sense that it need
5 not be the actual number of p27 protein molecules or moles of p27 protein in the sample. Rather, the amount determined may merely correlate with this number.

The subject invention further provides a method for
10 quantitatively determining the level of expression of p27 in a cell population, and a method for determining whether an agent is capable of increasing or decreasing the level of expression of p27 in a cell population. The method for
15 determining whether an agent is capable of increasing or decreasing the level of expression of p27 in a cell population comprises the steps of (a) preparing cell extracts from control and agent-treated cell populations, (b) isolating p27 from the cell extracts (e.g., by affinity chromatography on, and elution from, a cyclin E-Cdk2 complex
20 solid phase affinity adsorbant), (c) quantifying (e.g., in parallel) the amount of p27 inhibitor activity in the control and agent-treated cell extracts using a cyclin E-Cdk2 kinase assay (e.g., histone H1 assay described infra). Agents that induce increased p27 expression may be
25 identified by their ability to increase the amount of p27 inhibitor activity in the treated cell extract in a manner that is dependant on transcription, i.e., the increase in p27 inhibitor activity is prevented when cells are also treated with an inhibitor of transcription (e.g.,
30 actinomycin D). In a similar manner, agents that decrease expression of p27 may be identified by their ability to decrease the amount of p27 inhibitor activity in the treated cell extract in a manner that is dependent upon
transcription.

35

The subject invention further provides a method of determining whether a cell sample obtained from a subject possesses an abnormal amount of p27 protein which comprises (a) obtaining a cell sample from the subject, (b) 5 quantitatively determining the amount of p27 protein in the sample so obtained, and (c) comparing the amount of p27 protein so determined with a known standard, so as to thereby determine whether the cell sample obtained from the subject possesses an abnormal amount of p27 protein.

10

The subject invention further provides a method of determining whether the amount of p27 protein in a cell sample obtained from a subject having a disease is correlative with the disease which comprises determining 15 whether a cell sample obtained from the subject possesses an abnormal amount of p27 protein, an abnormal amount of p27 protein in the sample indicating that the amount of p27 protein in the cell sample obtained from the subject having the disease is correlative with the disease.

20

The subject invention further provides a method of quantitatively determining the specific activity of p27 protein in a sample which comprises quantitatively determining (i) the ability of the p27 protein in the sample 25 to inhibit the activation of cyclin E-Cdk2 complex and (ii) the total amount of p27 protein in the sample, and dividing the ability of the p27 protein so determined by the total amount of p27 protein so determined so as to thereby quantitatively determine the specific activity of p27 30 protein in the sample.

The subject invention further provides a kit for practicing the methods of the subject invention. In one embodiment, the kit comprises suitable amounts of p27 protein, cyclin E 35 and Cdk2. The kit may further comprise suitable buffers,

and a package insert describing p27 as an inhibitor of cyclin E-Cdk2 complex activity.

5 The subject invention further provides a method of diagnosing a hyperproliferative disorder in a subject which disorder is associated with the presence of a p27 protein mutation in the cells of the subject, which comprises determining the presence of a p27 protein mutation in the cells of the subject, said mutation being associated with a
10 hyperproliferative disorder, so as to thereby diagnose a hyperproliferative disorder in the subject.

As used herein, "diagnosing" means determining the presence of a hyperproliferative disorder in a subject. In one
15 embodiment, "diagnosing" additionally means determining the type of hyperproliferative disorder in a subject.

As used herein, a "p27 protein mutation" may be any abnormality in the primary sequence of p27 protein resulting
20 from an abnormality in the genomic DNA sequence encoding same or controlling the expression of same. For example, the p27 protein mutation may be a point mutation, a deletion mutation of a portion of p27 protein, or an absence of the entire p27 protein resulting from an abnormality in the
25 structural gene encoding same or regulatory DNA sequence controlling the expression of same.

Determining the presence of a p27 protein mutation may be accomplished according to methods well known to those
30 skilled in the art. Such methods include probing a subject's DNA or RNA with a p27 nucleic acid probe. Such methods also include analyzing a protein sample from the subject for p27 protein structural abnormalities or functional abnormalities resulting therefrom.

In the preferred embodiment, the subject is a human and the hyperproliferative disorder is cancer.

5 The subject invention further provides a pharmaceutical composition which comprises an effective amount of a recombinant virus capable of infecting a suitable host cell, said recombinant virus comprising the nucleic acid molecule of the subject invention, and a pharmaceutically acceptable carrier.

10

The "suitable host cell" is any cell in which p27 protein would normally be produced in a healthy subject.

15 Pharmaceutically acceptable carriers are well known to those skilled in the art and include, but are not limited to, 0.01-0.1M and preferably 0.05M phosphate buffer or 0.8% saline. Additionally, such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous
20 solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media.
25 Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preserva-
30 tives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases and the like.

Finally, this invention provides a method for treating a
35 subject suffering from a hyperproliferative disorder

associated with the presence of a p27 protein mutation in the cells of the subject, which comprises administering to the subject an amount of the pharmaceutical composition of the subject invention effective to treat the subject.

5

In the preferred embodiment, the subject is a human and the hyperproliferative disorder is cancer.

In order to facilitate an understanding of the Experimental
10 Details section which follows, certain frequently occurring methods and/or terms are best described in Sambrook, et al. (1989).

This invention will be better understood by reference to the
15 Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

I

5 Summary

Cell-cell contact and TGF- β can arrest the cell cycle in G1. Mv1Lu mink epithelial cells arrested by either mechanism are incapable of assembling active complexes containing the G1
10 cyclin, cyclin E, and its catalytic subunit, Cdk2. These growth inhibitory signals block Cdk2 activation by raising the threshold level of cyclin E necessary to activate Cdk2. In arrested cells the threshold is set higher than physiological cyclin E levels, and is determined by an
15 inhibitor that binds to cyclin E-Cdk2 complexes. A 27 kD protein that binds to and prevents the activation of cyclin E-Cdk2 complexes can be purified from arrested cells, but not from proliferating cells, using cyclin E-Cdk2 affinity chromatography. p27 is present in proliferating cells, but
20 it is sequestered and unavailable to interact with cyclin E-Cdk2 complexes. Cyclin D2-Cdk4 complexes competitively bind to and down-regulate the activity of p27 and may thereby act in a pathway that reverses Cdk2 inhibition and enables G1 progression.

25

Methods

Cell culture

Exponentially growing Mv1Lu cells were growth arrested by
30 culturing them to confluence in the presence of 10% fetal bovine serum. Cells were released from contact inhibition by trypsinization and reseeding in sparse conditions. TGF- β (100pM) was added to the cells at the indicated times. Cell entry into S phase was routinely confirmed by measuring 125 I-

deoxyuridine incorporation into DNA (Laiho et al., 1990).

Preparation of recombinant proteins

Cyclin E, Cdk2, Cdk2-HA, and Cdk2K were prepared by the
5 method of Desai et al (1992). Briefly, 100 mm plates of
confluent Sf9 cells were infected with the appropriate
baculovirus at an m.o.i. of 5-20 p.f.u per cell. After 48
hours of infection the cells were collected and lysed by
Dounce homogenization or cup-horn sonication in hypotonic
10 buffer. The extract is clarified by ultracentrifugation and
stored at -70° C. The baculoviral vectors containing cyclins
D1, D2, D3, Cdk4 and catalytically inactive Cdk4 have been
previously described (Matsushime et al., 1992; Kato et al.,
1993).

15

Purification and Microsequencing of p27^{Kip-1}

In order to purify p27 in amounts sufficient for
microsequence analysis, the purification protocol used as
the starting material 200 15-cm dishes containing confluent
20 cultures of contact inhibited Mv1Lu cells ($\sim 2 \times 10^{10}$ cells).
Cells were lysed by sonication in 33 ml of hypotonic
extraction buffer, and cell debris were removed by
centrifugation at 200,000 x g for 1 hour. The lysate was
heated to 100 °C for 5 min, and precipitated material was
25 removed by centrifugation at 100,000 x g for 15 min. The
supernatant was adjusted to NP-40 lysis buffer conditions
(Polyak et al., 1994) with 4x NP-40 lysis buffer, and
precleared by two successive 30 min. incubations with 5 ml
of agarose at 4 °C and once with 5 ml of nickel-NTA-agarose
30 under the same conditions.

The precleared lysate was allowed to bind to an affinity
column for 2 hours at 4 °C. This affinity column consisted
of nickel-NTA-agarose containing baculoviral Cdk2 in complex

with baculoviral cyclin E tagged at the N-terminus with a hexahistidine sequence that allows binding to nickel-NTA-agarose. The column was washed once with 50 ml of NP-40 lysis buffer, and five times with 50 ml of SDS/RIPA buffer
5 at room temperature. Bound proteins were eluted with 5 ml of a Hepes-buffered solution (pH 7.0) containing 6M guanidium hydrochloride. The eluate was dialyzed overnight against Hepes-buffered solution, and proteins were precipitated with 4 volumes of acetone at -20 °C for 30 min.
10 Precipitated proteins were collected by centrifugation at 20,000 x g for 15 min, solubilized in SDS electrophoresis sample buffer containing dithiothreitol, and electrophoresed on a 12% polyacrylamide gel. After electrophoresis, the gel was blotted onto nitrocellulose at 35V overnight in
15 Tris/glycine/methanol transfer buffer.

The nitrocellulose membrane was stained with Ponceau stain to detect proteins. According to this assay, the filters contained only two proteins that were well separated from
20 each other and were of 27 kd and 34 kd, respectively. These proteins were identified as p27 and p34^{cdk2}. Two separate preparations gave similar results. The yield of p27 in these two preparation was approximately 0.3 µg and 1 µg, respectively, as estimated by from the Ponceau staining and
25 from microsequencing.

The protein of nitrocellulose containing purified p27 was excised and subjected to tryptic digestion in preparation for microsequencing analysis. After HPLC of the tryptic
30 digests, the following peptides were sequenced:

1. Asn-Leu-Tyr-Pro-Leu-Thr-Asn-Tyr-Thr-Phe
2. Thr-Asp-Thr-Ala-Asp-Asn-Gln-Ala-Gly-Leu-Ala-Glu-Gln
3. Gln-Ala-Val-Pro-Leu-Met-Gly-Pro-Gln-Glu

4. Leu-Pro-Glu-Phe-Tyr-Tyr-Arg-Pro-Pro-Arg-Pro-Pro
 5. Tyr-Glu-Trp-Gln-Glu-Val

No similarity has been found between these sequences and
 5 protein sequences deposited in Genbak, EMBL Data Library,
 Brookhaven Protein Databank, Swiss Prot or PIR databases,
 according to the updates available in December 31, 1993.

Oligonucleotides for obtaining p27 cDNA

10 Oligonucleotides to be used in obtaining the full-length
 cDNA sequence of p27 are shown in Table II:

Table II

15 Peptide #1: None

Peptide #2:

Sense 5' - AC(N) - GA(T/C) - AC(N) - GA(T/C) - AA(T/C) -
 CA(A/G) - GC-3'

20

Antisense 5' - (N)GC - (T/C)TG - (A/G)TT - (A/G)TC - (N)GC -
 (N)GT - (A/G)TC - (N)GT-3'

Peptide #3:

25

Sense 5' - CA(A/G) - GC(N) - GT(N) - CC(N) - CT(N) - ATG-GG-3'

and 5' - CA(A/G) - GC(N) - GT(N) - CC(N) - TT(A/G) - ATG
 -GG-3'

30

Antisense 5' - (N)CC - CAT - (N)AG - (N)GG - (N)AC - (N)GC -
 (T/C)TG-3'

35

and 5' - (N)CC - CAT - (T/C)AA - (N)GG - (N)AC - (N)GC -
 (T/C)TG-3'

Peptide #4:

40 Sense 5' - CC(N) - GA(A/G) - TT(T/C) - TA(T/C) - TA(T/C) -
 (C/A)G-3'

Antisense 5' - C(T/G) - (A/G)TA - (A/G)TA - (A/G)AA - (T/C)TC -
 (N)GG-3'

45 Peptide #5:

Sense 5' - TA(T/C) - GA(A/G) - TGG-CA(A/G) - GA(A/G) - GT-3'
Antisense 5' - (N)AC- (T/C)TC- (T/C)TG-CCA- (T/C)TC-
(A/G)TA-3'

5

CAK

CAK was purified from *Xenopus* egg extracts through the Mono Q step exactly as described (Solomon et al., 1993) and was
10 used at a final concentration of 1-2 units per ml.

Metabolic labeling

Mv1Lu cultures in 150 mm dishes were incubated for 30 minutes in methionine-free medium supplemented with 10%
15 dialyzed fetal bovine serum, followed by incubation for 2 hours in the same medium with 200 μ Ci/ml of 35S-methionine (Trans 35S label, ICN). Cells were collected by trypsinization and centrifuged at 2000g for 5 minutes. Cell pellets were lysed by gentle agitation for 30 minutes at 4°C in 10
20 volumes of NP40 lysis buffer (50 mM Tris HCl pH 7.4, 200 mM NaCl, 2 mM EDTA, 0.5% NP40, 0.3 mM Na-orthovanadate, 50 mM NaF, 80 μ M b-glycerophosphate, 20 mM Na pyrophosphate, 0.5 mM DTT and protease inhibitors) and lysates were clarified by centrifugation (10,000g 15 minutes at 4°C). Prior to
25 binding reactions the supernatants were precleared twice with sepharose and once with protein A-sepharose.

Cdk activation assays

Indicated amounts of baculovirus expressed recombinant
30 cyclin, Cdk, or cyclin-Cdk complex were added to 50 micrograms of extracts prepared by sonication in a hypotonic buffer as previously described (Koff et al 1993). In all cases the exogenous cyclins and Cdks were added in the form of an unfractionated Sf9 cell lysate. Cyclins and Cdks
35 typically comprise at least 1-3% of total cell protein. Uninfected Sf9 cell lysates have been tested in all assays

and have no activity. After 30 minutes at 37° C the reaction was adjusted to 0.5% NP40, 250 mM NaCl and immunoprecipitated with the indicated antibody. Immunoprecipitates were subsequently assayed for histone H1 kinase activity as described (Koff et al. 1993). For experiments in which the effect of the D cyclins and Cdk4 on cyclin E activity were tested, all cyclins and Cdks were added to the cell extract together. Heat treatment of extracts was performed by incubating extracts to 100°C for 5 minutes. Coagulated protein was then pelleted by microcentrifugation. For experiments in which Cdk2 immunoprecipitates were tested for activation by cyclin E, 20 µl of antiserum to the C-terminus of CDK2 (Koff et al, 1993) was adsorbed to protein A sepharose and washed into NP40 RIPA buffer. 300µg of extract was subsequently incubated with the anti-CDK2 sepharose for 90 minutes at 4°C. The precipitate was washed twice with NP40 RIPA buffer and 4 times with buffer A containing 10 mM ATP. Cyclin E and CAK were added as described below and reactions were incubated for 30 minutes at 37°C and subsequently assayed for H1 kinase activity.

Inhibitor depletion

Cyclin E-Cdk2 sepharose was prepared by mixing 1.2 µl of Sf9 cell lysate containing Hemagglutinin tagged Cdk2 (Cdk2-HA) with 12 µl lysate containing cyclin E in buffer A (30 mM HEPES-KOH pH 7.5, 7.5mM MgCl₂, 1 mM DTT) containing 10 mM ATP and incubated at room temperature for 30 minutes to allow complete formation. The assembly reaction was then adjusted to 250 mM NaCl and 0.5% NP40. The Cdk2-HA containing complexes were immunoprecipitated with the 12CA5 monoclonal antibody (BABC0) and collected on protein A-sepharose. Cdk2 sepharose was prepared in an identical manner except cyclin E was omitted. Immunoprecipitates were washed twice with NP40 RIPA buffer (0.5% NP40, 250 mM NaCl,

10 mM EDTA, 20 mM Tris-HCl pH 7.4) and four times with buffer A. The matrix was divided into 4 aliquots and incubated with 100 μ g of cell extract in buffer A containing 3 mM ATP, 20 μ g/ml creatine phosphokinase, 40 mM phospho-
5 creatine for 45 minutes at 37°C. After incubation the supernatant was collected and assayed for Cdk2 activation by addition of recombinant cyclin E as described below. A critical parameter in the execution of this experiment is to ensure that no cyclin, Cdk or complex leaks from the beads
10 into the cell extract. This is unpredictable and must be checked by immunoblotting each time the experiment is performed.

Cyclin E-Cdk2 binding assays

15 Complexes of baculoviral cyclin E with baculoviral Cdk2 containing the influenza virus Hemagglutinin epitope HA1 were formed as described below. The complexes were immunoprecipitated in NP40-RIPA buffer (50 mM Tris-HCl pH = 7.4, 250 mM NaCl, 0.5% NP-40, 50 mM NaF, 0.3 mM Na-ortho-
20 vanadate, 5 mM EDTA and protease inhibitors) with anti-HA monoclonal antibody (12CA5, BabCo) and bound to protein A - sepharose. Cdk2 or cyclin E-Cdk2 adsorbed to protein A-sepharose were incubated with metabolically labeled cell lysates from 10^7 cells for 2 h at 4°C. Unless otherwise
25 indicated, the beads were washed several times with SDS-RIPA buffer, and the proteins were eluted by heating in SDS-PAGE sample buffer and analyzed on 12% polyacrylamide gels followed by fluorography. For heat treatment metabolically labeled cell lysates were heated for 3 minutes at 100 °C,
30 the precipitated proteins were removed by microcentrifugation and the clarified lysates were incubated with protein A-Sepharose bound Cdk2 or cyclin ECdk2 complexes. In binding assays using cyclin D2-Cdk4 complexes, metabolically labeled cell extracts were pre-incubated with 4 μ l of cyclin D2-Cdk4

complex for 30 minutes at 4°C before addition of protein A-sepharose bound Cdk2 or cyclin E-Cdk2. After removing the sepharose beads, cell extracts were immunoprecipitated with Cdk4 antiserum and the immunoprecipitates were analyzed on
5 12% SDS-PAGE.

Affinity purification of p27 and denaturation-renaturation experiments

HA-tagged Cdk2, alone or in complex with cyclin E, was bound
10 to HA antibody immobilized on protein A sepharose beads (ImmunoPure Orientation Kit, Pierce,) and used to isolated proteins from metabolically labeled cell lysates. Bound proteins were eluted from the column in 0.1M glycine pH 2.8 and precipitated with 4 volumes of ice-cold acetone and kept
15 at -20°C for 20 minutes. The precipitates collected by microcentrifugation for 30 minutes were washed several times with cold acetone, and dissolved in 6M guanidium chloride in 1 x HBB buffer (25 mM HEPES-KOH pH 7.7, 25 mM NaCl, 5 mM MgCl₂, 0.05% NP-40, 1mM DTT). For renaturation (Kaelin et
20 al., 1992), samples were dialyzed overnight against 1 x HBB buffer and used either in kinase inhibition assays or for binding to cyclin E-Cdk2 sepharose. For cyclin E associated H1 kinase inhibition assays, aliquots (37.5 μ l of protein) from 100,000 x g supernatants of lysates prepared from
25 exponentially growing Mv1Lu cells, were incubated for 30 min. at 37 °C with physiological amounts of baculoviral cyclin E either alone or in the presence of the indicated volumes of renatured eluates. After incubation, samples were precipitated with cyclin E antiserum, and assayed for
30 histone H1 kinase activity. The relative cyclin E-associated H1 kinase activity was quantitated using a Molecular Dynamics Phosphorimager ImageQuant software.

To assay the activity of protein eluted from gel slices

cyclin E Cdk2-HA affinity column eluates were run on 12% polyacrylamide gels along with molecular weight markers (Amersham). Part of the sample was run on the same gel, stained with Commassie, destained and detected by
5 fluorography. The gel was cut as indicated (between 0.5 to 1cm/slice) and the proteins were isolated from the gel as described (Boyle et al., 1991). The isolated proteins were renatured and used for kinase inhibition assays as described below.

10

Results

Non-proliferating cells contain an inhibitor of Cdk2 activation

15 Cell free extracts from contact inhibited, TGF- β arrested and proliferating cells were used to investigate the mechanism that blocks activation of the cyclin E-Cdk2 complex. It has been shown that addition of physiological amounts of cyclin E to these cell extracts resulted in an
20 increase in the amount of immunoprecipitable cyclin E-Cdk2 complexes; however, only the cyclin E-Cdk2 complexes assembled in extracts from proliferating cells were enzymatically active using histone H1 as a substrate (Koff et al, 1993; see also Figure 1A). Cell extracts, therefore,
25 recapitulate the block to Cdk2 activation observed in intact cells.

The block to Cdk2 activation in extracts from non-proliferating cells could be overcome by addition of cyclin E
30 protein to greater than physiological levels (Figure 1A). Cyclin E was expressed in Sf9 cells using a baculoviral expression vector and the amount of cyclin E in Sf9 extracts was compared to that in Mv1Lu cell extracts by immuno-
blotting (not shown). In the experiment illustrated in
35 Figure 1A, 0.05 μ l of Sf9 lysate contained as much cyclin E

as 50 μ g of total cell protein from Mv1Lu cell lysates. Addition of cyclin E (in the form of Sf9 lysate) to an extract from proliferating cells gave a linear increase in cyclin E-associated histone H1 kinase activity (proliferating cells were harvested 15 hours after release from contact inhibition, at which time they were in early S phase). In contrast, titration of up to 3 times physiological levels of cyclin E into extracts from contact inhibited or TGF- β treated cells resulted in no increase in immunoprecipitable cyclin E-associated kinase activity. As more cyclin E was added, cyclin E-associated kinase activity became detectable and increased in proportion. Thus, extracts from non-proliferating cells demonstrated an elevated threshold level of cyclin E necessary to activate Cdk2. Contact inhibited cells appeared to have a higher threshold than cells arrested in G1 by exposure to TGF- β , but in both cases the cyclin E requirement was substantially greater than the physiological levels of cyclin E achieved in proliferating cells.

Supra-physiological amounts of cyclin E were required to activate Cdk2 in extracts from non-proliferating cells. This could not be explained by lower levels of Cdk2 or cyclin E, nor did these cells appear to lack other factors necessary for Cdk2 activation (Koff et al., 1993; see below). One explanation was that non-proliferating cells contained a titratable inhibitor of Cdk2 activation. Mixing experiments supported this conclusion. Extracts from proliferating cells were mixed with those from either contact inhibited or TGF- β treated cells. Physiological levels of cyclin E were added to the mixed extracts and then cyclin E and any associated kinases were immunoprecipitated using antibodies to the cyclin. Identical results were obtained using an anti-Cdk2 antiserum (not shown). In mixed extracts cyclin E-associated kinase activity was reduced below that recovered from

extracts of proliferating cells alone (Figure 1B). Thus, extracts from non-proliferating cells contained an excess of an inhibitor of Cdk2 activation. Note that extracts from contact inhibited cells had both a higher cyclin E
5 activation threshold and a greater inhibitory effect in mixing experiments than extracts from TGF- β treated cells. However, the abundance of the Cdk2 inhibitory activity depended upon the duration of exposure to TGF- β . For instance, it is shown that an extract from cells exposed to
10 TGF- β for 6 hours beginning in late G1 did not contain sufficient inhibitory activity to block Cdk2 activation when mixed with an extract from proliferating cells (Koff et al., 1993) and cells exposed to TGF- β for 48 hours had more inhibitory activity than cells exposed for 15 hours (not
15 shown).

A Cdk2 inhibitor binds to cyclin E-Cdk2 complexes

The inhibitor of Cdk2 activation present in extracts from non-proliferating cells could be depleted using a cyclin
20 E-Cdk2 affinity matrix. Cyclin E-Cdk2 complexes were formed by mixing extracts from Sf9 cells infected with baculoviral vectors expressing either Cdk2 tagged with an influenza virus hemagglutinin (HA) epitope or cyclin E. Although neither extract alone contains significant H1 kinase
25 activity, mixing of the extracts yields high levels of active enzyme (Kato et al., 1993). The cyclin E-Cdk2 (HA) complexes were immunoprecipitated with sepharose-linked monoclonal antibody directed against the HA tag on Cdk2. Control immunoprecipitations were performed using the
30 monoclonal antibody beads alone. Cell extracts were incubated with either the cyclin E-Cdk2 beads or the control beads, and after pelleting the supernatants were assayed for the ability of exogenously added cyclin E to activate endogenous Cdk2. After depletion of cyclin E-Cdk2 binding
35 proteins, cyclin E was able to activate Cdk2 almost equally

in extracts from proliferating and non-proliferating cells (Figure 2A). Immunoblotting showed that this protocol had no effect on the levels of either cyclin E or Cdk2 in the cell extracts (not shown). In this experiment some stimulatory effect of depleting cyclin E-Cdk2 binding proteins was also observed in extracts from proliferating late G1 cells, suggesting they are not completely devoid of the inhibitor (see below). Complexes containing cyclin E and a catalytically inactive mutant of Cdk2 also were able to sequester inhibitory activity when added directly to cell extracts (see Figure 2C). Thus reversal of inhibitory activity did not require phosphorylation by the added cyclin E-Cdk2 complexes. These experiments showed that the inhibitor of Cdk2 activation bound to cyclin E-Cdk2 complexes.

In parallel it is observed that beads containing just Cdk2 alone were unable to deplete the inhibitory activity from cell extracts (Figure 2A). This experiment suggested that the inhibitor bound to cyclin E-Cdk2 complexes but not to Cdk2 alone. To directly test this idea, Cdk2 was immunoprecipitated from extracts of proliferating, contact inhibited and TGF- β treated cells. In all cases, the immunoprecipitated Cdk2 protein could be activated by addition of both cyclin E and p34cdc2 Activating Kinase (CAK) (Figure 2B). Thus, the Cdk2 protein in non-proliferating cells was not intrinsically incapable of activation, nor was it tightly associated with an inhibitor of activation.

Since the Cdk2 inhibitor could bind to cyclin E-Cdk2 complexes, but not to Cdk2, it appeared to recognize either the cyclin-Cdk complex or cyclin. Cyclin E-Cdk2 complexes were more effective at removing the inhibitory activity than was cyclin E, suggesting that the inhibitor interacted

preferentially with complexes. Cyclin E was added to a non-proliferating cell extract at a level below the threshold necessary to activate Cdk2 (Figure 2C). The assembly of additional cyclin-Cdk2 complexes was then induced by supplementing the extracts with an exogenous Cdk2 protein that was rendered catalytically inactive by a mutation of its ATP binding site (Gu et al., 1992). In the absence of extra Cdk2 no kinase activity was detected in cyclin E immunoprecipitates. When extracts were supplemented with catalytically inactive Cdk2, cyclin E regained H1 kinase activity as a result of activating the endogenous Cdk2. Thus, the cyclin E threshold for Cdk2 activation could be lowered by assembling additional cyclin-Cdk complexes while keeping the total amount of cyclin E constant.

The inhibitor is neither an anti-CAK nor a tyrosine kinase
Previous experiments (Koff et al., 1993) indicated that cyclin E-Cdk2 complexes formed in extracts from non-proliferating cells were not phosphorylated at an essential threonine residue (Gu et al., 1992; Solomon et al., 1992) possibly accounting for their inactivity. This raised the possibility that CAK was a target of the inhibitor. This initially seemed unlikely because the inhibitor bound directly to the cyclin E-Cdk2 complex. This idea was reconsidered in light of recent evidence that CAK is itself a distant member of the Cdk protein family (Fesquet et al., 1993; Poon et al., 1993; Solomon et al., 1993) and therefore might also bind to the inhibitor. Previous work, in another system, indicated that activation of the cyclin B-Cdc2 complex was not blocked by the Cdk2 inhibitor (see below). Cyclin B and Cdc2 were therefore used to assay CAK activity, given that CAK is also required to activate the cyclin B-Cdc2 complex (Solomon et al., 1990).

Cdc2 was activated equally when cyclin B was added to

extracts from either proliferating cells or TGF- β arrested cells (Figure 3A). Therefore, functional CAK was present in extracts from TGF- β treated cells. CAK was limiting in this experiment since addition of purified CAK to these extracts catalyzed the activation of additional cyclin B-Cdc2 complexes (Figure 3B). Moreover, the activity of the added CAK was similar in extracts from TGF- β treated and proliferating cells (Figure 3B). Thus, exogenous CAK was not inhibited. Control experiments showed that this CAK was able to activate cyclin E-Cdk2 complexes when they were assembled by mixing Sf9 cell lysates containing cyclin E and Cdk2 expressed from baculoviral vectors (Solomon et al, 1993; data not shown). However, the added CAK did not change the threshold level of cyclin E required to activate Cdk2 (not shown). Thus, the inhibitor neither blocked CAK nor could its effects be overcome by excess CAK. Inhibition of CAK was not sufficient to explain the block to Cdk2 activation.

To determine if tyrosine phosphorylation contributed to the inhibition of Cdk2 activity, cyclin E was added to non-proliferating cell extracts at sub-threshold levels and the cyclin E-Cdk2 complexes were immunoprecipitated using anti-cyclin E antibodies. No tyrosine phosphorylation of Cdk2 in the inactive cyclin E-Cdk2 complexes was detected by immunoblotting with anti-phosphotyrosine antibodies (not shown). As a positive control, phosphotyrosine was readily detected in Cdc2 immunoprecipitated from human cells.

Cyclin D2-Cdk4 complexes facilitate Cdk2 activation

As cells traverse G1, complexes between Cdk4 and the D-type cyclins appear prior to the formation of active complexes containing cyclin E and Cdk2 (reviewed in Sherr, 1993). Contact inhibited Mv1Lu cells do not express significant levels of cyclin D1 or D2 (not shown) and Cdk4 synthesis is repressed in cells arrested in G1 by exposure to TGF- β (Ewen

et al., 1993; K.P and J.M., unpublished observations; see also Figure 5D). Thus, accumulation of cyclin D-Cdk4 complexes is limiting in G1 arrested cells. These observations suggested that cyclin D-Cdk4 complexes could potentially have a role in removing the Cdk2 inhibitor during cell cycle progression. Indeed, Ewen et al. (1993b) recently showed that constitutive ectopic expression of Cdk4 can override the TGF- β block to Cdk2 activation and cell cycle progression. This phenomenon was tested by asking whether the restoration of cyclin D-Cdk4 complexes to extracts from non-proliferating cells might overcome the block to Cdk2 activation.

Cdk4 is a partner of the D-type cyclins and does not form active complexes with cyclins E, A or B. It interacts equally well with each of the D-type cyclins when they are co-expressed in insect cells. Cyclin D-Cdk4 complexes are poorly active on histone H1 but show strong activity using the Rb protein as substrate (Matsushime et al., 1992; Kato et al., 1993). Complexes between Cdk4 and either cyclin D1, D2 or D3 were assembled by co-infection of Sf9 cells with baculoviral vectors and Sf9 lysates were added to extracts from proliferating and non-proliferating Mv1Lu cells. Sub-threshold amounts of cyclin E were then added, and activation of Cdk2 was tested after immunoprecipitation of cyclin E-Cdk2 complexes with antibodies to cyclin E. Addition of cyclin D2-Cdk4 complexes, but neither subunit alone, to extracts from contact inhibited and TGF- β arrested cells allowed cyclin E to activate Cdk2 to a level equivalent to that observed in extracts from proliferating cells (Figure 4A). Titrations demonstrated that the amount of cyclin D2-Cdk4 necessary to block the Cdk2 inhibitor was less than that present in an equivalent amount of extract from proliferating cells (not shown). In contrast, the activity of cyclin E was not increased when cyclin D2-Cdk4

complexes were added to extracts from proliferating cells. Moreover, the cyclin D2-Cdk4 complex did not have CAK activity, since it was unable to substitute for CAK in promoting the activation of cyclin E-Cdk2 complexes assembled from proteins expressed in Sf9 cells (not shown). Thus, the cyclin D2-Cdk4 complex reversed the inhibition of Cdk2 activation. Equal amounts of cyclin D1-Cdk4 and cyclin D3-Cdk4 complexes, as estimated by immunoblotting of Sf9 lysates, were much less effective in lowering the cyclin E threshold for Cdk2 activation (Figure 4B). The inability of cyclin D1 or cyclin D3-Cdk4 complexes to sequester the Cdk2 inhibitor was not because those complexes were unstable in cell lysates (not shown).

Quite surprisingly, the ability of cyclin D2-Cdk4 to reverse Cdk2 inhibition did not require Cdk4 catalytic activity. Complexes formed between cyclin D2 and a catalytically inactive mutant Cdk4 subunit were as effective as enzymatically active cyclin D2-Cdk4 complexes in removing the Cdk2 inhibitor (Figure 4A). Titrations with different amounts of cyclin D2 complexes containing either catalytically active or inactive Cdk4 revealed that their specific activities in reversing the Cdk2 inhibition were very similar (not shown). This ruled out the possibility that Cyclin D2-Cdk4 must phosphorylate the inhibitor to inactivate it, and excluded any model in which cyclin D2-Cdk4 bypassed the inhibitor by functioning as a CAK. It, therefore, seemed likely that cyclin D2-Cdk4 removed the Cdk2 inhibitor by binding to it directly and sequestering it from Cdk2 (see below).

The Cdk2 inhibitor is a 27 kd protein

The above observations indicated (i) that a functional cyclin E-Cdk2 inhibitor was present in extracts from contact inhibited cells or cells released from contact inhibition in

the presence of TGF- β , but not in extracts from proliferating cells; (ii) that this molecule preferentially associated with cyclin E-Cdk2 complexes as opposed to either subunit alone; and (iii) that it could be depleted by preincubation of cell extracts with catalytically active or inactive cyclin D2-Cdk4 complexes. To identify a factor that might display these properties, Mv1Lu cells were metabolically labeled with 35S-methionine, and lysates were incubated with Sepharose beads that contained immunoadsorbed recombinant Cdk2, either alone or in complexes with recombinant cyclin E. Denatured 35S-labeled proteins, eluted by heating the beads with buffer containing 1% SDS, were visualized by gel electrophoresis and fluorography (Figure 5A). All cell lysates yielded a similar pattern of cyclin E-Cdk2 binding proteins with the exception of a 27 kd protein that was recovered from extracts of contact-inhibited or TGF- β inhibited cells, but not late G1 phase cells (Figure 5A). This protein, referred to as p27, was isolated using cyclin E-Cdk2 complexes but not Cdk2 alone (Figure 5A). The recovery of p27 increased in proportion to the amount of cyclin ECdk2 complex used until it reached a maximum (Figure 5B), indicating that binding of p27 to cyclin E-Cdk2 complexes was saturable. This was consistent with the observation that Cdk2 inhibitor activity could be depleted by cyclin E-Cdk2 complexes. As expected, stoichiometric amounts of p27 were also observed in cyclin E immunoprecipitates from growth arrested cells (not shown).

Cell extracts that received recombinant cyclin D2-Cdk4 complex no longer yielded p27 when the mixture was adsorbed to cyclin E-Cdk2-Sepharose (Figure 5C). After removal of the cyclin E-Cdk2-Sepharose beads from samples that received cyclin D2-Cdk4, the precleared supernatants were incubated with Cdk4 antibody to recover Cdk4 and its associated proteins. This yielded p34Cdk4 itself, whose levels were

highest in extracts from cells in late G1 and lowest in TGF- β treated cells (Figure 5D) (Matsushime et al., 1992; Ewen et al., 1993b). Using the same antiserum, Ewen et al., (1993b) used partial proteolytic digestion to confirm that
5 this is authentic Mv1Lu Cdk4. In addition, these immuno-precipitates contained a 27 kD protein in samples from contact-inhibited and TGF- β treated cells (Figure 5D). Lesser amounts of p27 were also recovered in Cdk4 immuno-precipitates from late G1 cell samples, even though p27
10 could not be recovered from those same extracts by cyclin E-Cdk2 affinity chromatography. This suggested that p27 was present in proliferating cells, but in a form unavailable to interact with exogenously added cyclin E-Cdk2 complexes (see below). Side-by-side comparison showed that p27 purified on
15 cyclin E-Cdk2 beads or by co-precipitation with Cdk4 had the same apparent molecular weight (not shown).

Experiments done to characterize the stability of this factor showed that heating cell extracts to 100°C for a
20 brief period preserved both the ability of p27 to bind to cyclin E-Cdk2 (Figure 6A) and the inhibitory activity as well (Figure 6C). Furthermore, when applied to extracts from cells in late G1 phase, heat treatment unexpectedly induced the appearance of both p27 (Figure 6A) and concomitantly
25 increased the level of Cdk2 inhibitory activity (Figure 6B). These results indicated that p27 and the inhibitory activity were both heat stable, and that they could be re-activated in late G1 extracts by a heat-sensitive mechanism. As expected, cyclin D2-Cdk4 complexes were also able to
30 sequester p27 from heat treated lysates (not shown).

Extracts from metabolically-labeled TGF- β treated cells were subjected to chromatography over cyclin E-Cdk2-sepharose or, as a control, Cdk2-sepharose. After washing, the beads were
35 eluted with an acidic buffer, and one portion of the eluate

was analyzed by SDS PAGE. This showed that p27 was the predominant labeled species recovered and was present only in the eluate from cyclin E-Cdk2 beads (Figure 7A). Samples from the same eluates were assayed for the presence of the Cdk2 inhibitor, and this activity was present in the eluate from cyclin E-Cdk2 beads but not Cdk2 beads (Figure 7B). The remainder of the eluate was concentrated by acetone precipitation, denatured in 6M guanidium hydrochloride, renatured by dialysis against isotonic buffer and subjected to a second round of binding to cyclin E-Cdk2 Sepharose. Elution from these beads by boiling in buffer containing 1% SDS yielded p27 as the only labeled band (Figure 7C). These results strongly supported the possibility that p27 and the inhibitory activity are one and the same. This conclusion was directly confirmed by fractionating the cyclin E-Cdk2 eluate by polyacrylamide gel electrophoresis and extracting the fractionated proteins from gel slices. Renatured proteins were tested for their ability to inhibit activation of Cdk2 by cyclin E (Figure 7D). The protein recovered from the gel slice containing p27 completely inhibited Cdk2 activation, and no additional inhibitory activity was recovered from any other gel slice.

Discussion

25

An inhibitor in non-proliferating cells was identified that prevents activation of complexes containing the G1 cyclin, cyclin E (Koff et al., 1991; Lew et al., 1991; Ohtsubo & Roberts, 1993), and its catalytic subunit, Cdk2 (Koff et al., 1992; Dulic et al., 1992). This inhibitory activity is, at least in part, attributable to a 27 kDa polypeptide, which has also been named p27^{Kip1} (Cdk inhibitory protein 1). The inhibitor and p27^{Kip1} share the following characteristics: they bind to cyclin E-Cdk2 complexes but not to Cdk2

alone; they are only detected in extracts from growth arrested cells; they can be sequestered by cyclin D2-Cdk4 complexes but not by either component alone; they are heat stable; they are latent in extracts of proliferating cells
5 but can be unmasked by brief heat treatment. In addition purified p27^{Kip1} inhibits Cdk2 activation by cyclin E when added to an extract from proliferating cells. While these data strongly suggest that p27^{Kip1} is at least a component of the Cdk2 inhibitor, it has not been determined whether
10 inhibition is due to p27^{Kip1} alone, or whether p27^{Kip1} recruits additional proteins to the cyclin E-Cdk2 complex. It has further been determined that p27, as well as cyclin E and Cdk2 are present in other organisms, e.g., mice and humans (data not shown).

15

The mechanism of p27^{Kip1} inhibition has features that distinguish it from pathways that control activation of the mitotic cyclin-Cdc2 complexes. First, p27^{Kip1} appears to act stoichiometrically rather than catalytically. Second,
20 tyrosine phosphorylation of Cdk2 was not detected in inactive cyclin E-Cdk2 complexes containing p27^{Kip1}, suggesting that p27^{Kip1} does not have tyrosine kinase activity or inhibit a tyrosine phosphatase. Complexes containing p27^{Kip1} were not efficiently phosphorylated by the p34Cdc2
25 activating kinase, CAK, and this might be sufficient to explain their inactivity. It is possible that p27^{Kip1} dephosphorylates Thr160, although it would be surprising if the enzymatic activity of a phosphatase were stable to heating to 100°C. It is more likely that binding of p27^{Kip1} to
30 the cyclin E-Cdk2 complex prevents Thr160 phosphorylation by altering the conformation of the T160 domain, or by sterically obstructing CAK. It would not be surprising if p27^{Kip1} functioned similarly to the negative regulatory subunits or domains of other protein kinases, perhaps even

interacting with the kinase active site as a pseudosubstrate.

It is intriguing that in addition to p27^{Kip1} other potential
5 regulators of Cdk activity during G1 also bind directly to
cyclin-Cdk complexes, including FAR1 (Peter et al., 1993),
p40 (Mendenhall, 1993), p16 and p21 (Xiong et al., 1992;
1993) and Rb (Kato et al., 1993; Dowdy et al., 1993; Ewen et
10 al., 1993a). While none of these other proteins has yet been
shown to directly inhibit Cdk activity, it seems likely that
at least some of them will perform this function. Direct
protein-protein interactions may be a way to focus
inhibitory signals on specific cyclin-Cdk complexes in a
cellular environment containing other more promiscuous
15 trans-acting regulators of Cdk activity.

p27^{Kip1} links growth inhibitory signals to cell cycle arrest
p27^{Kip1} was discovered in cells arrested in G1 by either
contact inhibition or TGF- β . A similar activity has also
20 been found to block Cdk2 activation in various cell types
deprived of specific growth factors, including serum-starved
fibroblasts and IL-2 deprived lymphocytes (unpublished
observations). Inhibition of Cdk2 activation by p27^{Kip1} or
functionally similar proteins, may be a general mechanism
25 through which diverse extracellular and intracellular
signals exert control on cell proliferation.

p27^{Kip1} constrains cell proliferation by setting the threshold
level of cyclin E necessary to activate Cdk2. If p27^{Kip1} acts
30 stoichiometrically, as these data suggest, then the Cdk2
activation threshold is reached soon after the amount of
cyclin E in the cell exceeds the amount of active p27^{Kip1}. In
arrested cells this threshold is set higher than
physiological cyclin E levels, and consequently only

inactive cyclin E-Cdk2 complexes assemble. The cyclin A-Cdk2 complex may be subject to similar control (Koff et al., 1993; Firpo et al., in preparation), and an inability to activate this complex should also contribute to cell cycle arrest (Girard et al. 1991; PacJano et al., 1992; 1993; Tsai et al., 1993).

How might growth inhibitory signals be linked to the activity of p27^{Kip1}? The simplest idea would be that growing cells do not contain much p27^{Kip1} and that signals which inhibit cell proliferation induce p27^{Kip1} synthesis or stabilization and thereby increase its amount above a critical basal level. This model can not be strictly correct because greatly increased quantities of p27^{Kip1} can be recovered from a latent pool once extracts from proliferating cells are subject to heat treatment. A substantial pool of p27^{Kip1} must be present in these extracts and must be sequestered by other molecules. This implies that p27^{Kip1} plays a normal role during the proliferative cell cycle and is not simply a response element for signals which induce growth arrest. The abundance of "free" p27^{Kip1} that is able to interact with the cyclin E-Cdk2 complex might, therefore, be modulated by an upstream regulator, such as the cyclin D2-Cdk4 complex, which also binds to p27^{Kip1} directly. This prevents association with cyclin E-Cdk2 and enables its functional activation, at least in vitro. The idea that p27 activity is governed by an upstream regulator does not exclude the possibility that the total cellular level of p27 may increase in arrested cells, and these experiments have not directly compared the total amounts of p27 in proliferating and arrested cells.

D-type cyclins are themselves targets of growth inhibitory signals (reviewed in Sherr, 1993). Their synthesis is

rapidly reduced in growth factor-deprived cells (Matsushime et al., 1991; Won et al., 1992; Kato et al., in press) and in contact inhibited cells (unpublished observations) leading to a reduction in cyclin D-Cdk4 levels (Matsushime et al., 1992). While D-type cyclin levels are not greatly affected by TGF- β blockade, TGF- β does reduce synthesis of Cdk4 so that a net reduction in cyclin D-Cdk4 complexes is achieved nevertheless (Ewen et al., 1993b). In TGF- β inhibited cells, where Cdk4 is limiting, expression of excess Cdk4 should lead to the formation of additional cyclin D-Cdk4 complexes and sequester p27^{Kip1}. In fact, enforced expression of Cdk4 in vivo reverses the block to Cdk2 activation in cells exposed to TGF- β (Ewen et al., 1993b). However, the addition of Cdk4 alone to extracts from TGF- β treated cells in vitro does not reverse the interaction of p27^{Kip1} with cyclin E-Cdk2. Unlike complexes with cyclin E and Cdk2, which can be formed in vitro by mixing the recombinant proteins produced in insect cells, D-type cyclins and Cdk4 do not assemble efficiently unless Sf9 cells are coinfecting with baculoviruses encoding both components (Kato et al., 1993). Although the reasons underlying these differences in complex formation have not been defined, all results are internally consistent and support the idea that cyclin D-Cdk4 complexes act upstream of cyclin E-Cdk2 by interacting with p27^{Kip1}. Although these ideas are based upon many observations made in intact cells, the proposed pathway containing cyclin D-Cdk4, p27^{Kip1} and cyclin E-Cdk2 has been tested directly only in vitro. One might expect that Cdk2 will be regulated by additional mechanisms, and that other novel Cdk complexes in addition to cyclin D2-Cdk4 could contribute to the titration of p27^{Kip1}.

It is not likely that the only role of cyclin D2-Cdk4 is to

titrate p27^{Kip1}, but rather, complex accumulation is likely to trigger the Cdk4-mediated phosphorylation of particular substrates necessary for G1 progression. Thus cyclin D complexed with catalytically inactive Cdk4 is sufficient to
5 sequester p27^{Kip1}, but is unlikely to fully substitute for all essential Cdk4 functions in vivo.

One feature of p27^{Kip1} induced cell cycle arrest is that cells can accumulate inactive cyclin E-Cdk2 complexes. Recovery
10 from cell cycle arrest, therefore, might not require synthesis of new cyclin E and assembly of new cyclin E-Cdk2 complexes. Rather the cell may make use of this latent pool of inactive complexes when cell proliferation resumes. This might be essential under circumstances where the signals
15 that promoted cyclin synthesis were transient, and absent when the growth inhibitory signals ceased. Thus far, however, conditions have not been defined that allow re-activation of inactive cyclin E-Cdk2p27^{Kip1} complexes. In vitro, only cyclin E-Cdk2 complexes which assemble after
20 titration of p27^{Kip1} are active, and the same may be true in vivo as well.

The presence of p27^{Kip1} in proliferating cells suggests that its role may not be restricted to inducing cell cycle arrest
25 in response to extracellular signals. It may also set the cyclin E threshold for execution of the G1 to S transition during each mitotic cycle. Cell fusion experiments have indicated that entry into S phase in mammalian fibroblasts is controlled by an activator that accumulates continuously
30 during G1 (Foumier and Pardee, 1975; Rao et al., 1977). By comparing the rate of S phase entry in mono- bi- and tri-nucleate cells it was concluded that the amount of this activator rather than its concentration was critical in determining the start of S phase. These observations are

consistent with a model in which the limiting step in Cdk2 activation is not assembly of the cyclin-Cdk2 complex, which should be concentration dependent, but instead involves the assembly of a sufficient number of complexes to overcome a threshold level of a stoichiometric inhibitor, such as p27^{Kip1}. It is also pointed out that spontaneous decay of p27^{Kip1} inhibited complexes to free p27^{Kip1} and active cyclin-Cdk2 might occur with first order (exponential) kinetics and could underlie the first order rate constants frequently reported for S phase entry in mammalian cells (Smith & Martin, 1973; Brooks et al., 1980).

p27^{Kip1} may enforce order during G1 progression

Cyclin-Cdk complexes appear in a specific order as cells transit G1 (Sherr, 1993). If it is assumed that this temporal order is essential for normal G1 progression, then cells must solve the problem of restoring order during recovery from cell cycle arrest. For example, contact inhibition and TGF- β interfere with the accumulation of cyclin D-Cdk4 complexes, but do not affect synthesis of cyclin E- and cyclin A-Cdk2 complexes, which act later in the cell cycle. If the cyclin E-Cdk2 and cyclin A-Cdk2 complexes were active during cell cycle arrest, then the normal order of Cdk action would be lost. p27^{Kip1} might ensure that this does not happen by preventing activation of these pre-existing complexes during cell cycle arrest. In addition, if the activity of p27^{Kip1} is itself controlled by cyclin D2-Cdk4, then this would provide an efficient mechanism for maintaining Cdk2 inactive until cyclin D-Cdk4 complexes assemble and execute their functions.

II

Experimental Procedures

- 5 Metabolic labeling, immunoprecipitations and peptide mapping
Mv1Lu cells were synchronized by contact inhibition, treated with TGF- β , metabolically labeled, lysed, immunoprecipitated with anti-Cdk2 antibody or chromatographed on cyclin E-Cdk2 affinity columns. For peptide mapping, the 27kd band present
10 in Cdk2 immunoprecipitates and in the cyclin E-Cdk2 affinity column eluates was cut out from the gels, digested with 0.1 μ g of V8 protease, and resolved on 15-22.5% gradient gels.

Baculoviral proteins

- 15 The human cyclin E cDNA (Koff et al., 1991) was tagged at the N-terminus with a hexahistidine sequence. This cDNA was cloned into baculovirus transfer vector pVL1392, and expressed in Sf9 cells as described in the BaculoGold Transfection Kit (Pharmingen). Baculoviral proteins were
20 prepared by the method of Desai et al., 1992.

Kip1 purification

- Two hundred 150mm dishes of contact inhibited Mv1Lu cells ($\sim 2 \times 10^{10}$ cells) were collected by trypsinization and lysed in
25 hypotonic buffer by sonication. The extracts were clarified by centrifugation, heated to 100°C for 5 min and clarified by centrifugation. Agarose-cleared extracts were allowed to bind to His-cyclin E-Cdk2 complexes immobilized on Ni⁺⁺-NTA-agarose. Specifically bound proteins were eluted with 6M
30 guanidium hydrochloride solution, dialyzed overnight against 1xHBB buffer (25 mM HEPES-KOH, pH 7.7, 150 mM NaCl, 5 mM MgCl₂, 0.05% NP-40 and 1 mM DTT) (Kaelin Jr et al., 1992) and acetone-precipitated.

Protein sequence analysis

Protein was fractionated by SDS-PAGE, electroblotted onto nitrocellulose, and the Ponceau S-stained 27 kDa band was excised and processed for internal amino acid sequence analysis (Tempst et al., 1990). HPLC peak fractions (over trypsin background) were analyzed by a combination of automated Edman degradation and matrix-assisted laser-desorption (MALDI-TOF) mass spectrometry. Mass analysis (on 2% aliquots) was carried out using a model LaserTec Research MALDI-TOF instrument (Vestec), and a-cyano-4-hydroxy cinnamic acid as the matrix. Chemical sequencing (on 95% of the sample) was done using an Applied Biosystems 477A sequenator optimized for femtomole level analysis.

15 Kip1 cDNA cloning and Northern blot analysis

RT-PCR reactions were performed using degenerate oligonucleotides as primers and total RNA from contact-inhibited Mv1Lu cells as template. The combination of one pair of primers (see Figure 9A) yielded a 135 bp fragment that was used to screen a 12APII cDNA library prepared from Mv1Lu cells. The mouse Kip1 cDNA was obtained from a 1EXlox mouse embryo cDNA library (Novagen), and the human Kip1 cDNA was obtained from a lgt11 kidney cDNA library (Clontech). Poly(A)⁺ RNA blots were hybridized with a PCR-derived fragment of the mouse Kip1 cDNA labeled by random priming.

In vitro translation

A NdeI-XhoI fragment containing the coding region of the mouse Kip1 cDNA (nucleotides 1-591) was subcloned to pCITE2a (Novagen). This construct encodes a fusion protein containing a C-terminal hexahistidine sequence and 6 amino acids from the vector at the N-terminus of Kip1. In vitro transcription and translation were performed using Red Nova lysate (Novagen).

Recombinant Kip1

A 591bp PCR generated NheI-XhoI fragment of the mouse Kip1 cDNA containing the full length coding region was subcloned into pET21a (Novagen), yielding a construct that encodes
5 Kip1 with a C-terminal hexahistidine sequence. The protein was expressed in BL21(DE3) bacteria and purified by sonicating cells in a solution containing 8M urea, 50 mM Tris-HCl (pH 7.4), 20 mM imidazole, clarified by centrifugation and bound to Ni⁺⁺-NTA agarose for 1 h at 4 °C. The
10 column was washed with a 6M to 0.75 M urea reverse gradient in 0.5M sodium chloride, 50 mM Tris (pH 7.4), and 20% glycerol and eluted with 200 mM imidazole, 20 mM HEPES pH 7.4, 1M KCl, 100 mM EDTA. The eluate was dialysed overnight against 1xHBB buffer and stored at -80°C until use.

15

In vitro kinase and Cdk2 activation assays

H5 insect cell extracts containing baculovirally-expressed cyclins and Cdks were incubated with recombinant Kip1 for 30 min at 37°C, precipitated with anti-HA antibody, and the
20 histone H1 kinase activity of these complexes was assayed (Koff et al., 1993). Rb kinase reactions were done according to Matsushime et al. (1991). The phosphorylation of the histone H1 band and Rb band were quantitated with Phosphorimager (Molecular Dynamics).

25

Hypotonic cell extracts from exponentially growing A549 cells were incubated with baculoviral His-cyclin E protein, with or without Kip1, at 37°C for 30 min. Mixtures were then diluted 10-fold in 1 x NP40 RIPA buffer containing 20 mM
30 imidazole and incubated with Ni⁺⁺-NTA-agarose at 4°C for 1h. One portion of the samples was run on 12% SDS-PAGE, and immunoblotted with anti-Cdk2 antibody (Koff et al., 1993).

Kip1 transfections and flow cytometry analysis

The mouse Kip1 cDNA (nucleotides -82 to +591) was subcloned into pCMV5 (Attisano et al., 1993). R-1B cells were cotransfected with 0.5 μ g/ml of pCEXV-3 containing murine CD16 cDNA (Kurosaki and Ravetch, 1989) and 3 μ g/ml of pCMV5 alone, or with 3 μ g/ml of pCMV5-Kip1 (Attisano et al., 1993). CD16 immunostained cells (Wirthmueller et al., 1992) were analyzed by flow cytometry using FACScan (Becton-Dickinson) and Multicycle software (PHOENIX Flow Systems).

10 Results

Purification and cloning of Kip1

Lysates from contact-inhibited Mv1Lu cells were heated to 100 °C, cleared of insoluble material, and allowed to bind to a cyclin E-Cdk2 affinity column. Elution with 6 M guanidium hydrochloride yielded recombinant Cdk2 released from the column, and the 27 kd protein Kip1. Dialyzed aliquots of this sample had strong inhibitory activity towards cyclin E-Cdk2 in histone H1 kinase assays, and this activity was shown to coelute from SDS-PAGE gel slices with Kip1. The Kip1 yield from two separate preparations ($\sim 2 \times 10^{10}$ cells each) was 0.3 μ g and 1 μ g, respectively.

In order to confirm that Kip1 interacts with Cdk2 in vivo, metabolically labeled extracts were immunoprecipitated from contact-inhibited Mv1Lu cells using anti-Cdk2 antibodies (Figure 8B). In addition to Cdk2, the precipitate contained a 27 kd band whose peptide map, after limited digestion with V8 protease, was identical to that of Kip1 purified from metabolically-labeled cells by cyclin E-Cdk2 affinity chromatography (Figure 8C). These results provided further evidence that the Cdk inhibitor purified by binding to cyclin E-Cdk2 in vitro was associated with Cdk2 in quiescent cells.

Various Kip1 tryptic peptide sequences were obtained by automated Edman degradation and used to design degenerate oligonucleotide primers for cDNA amplification by the reverse transcription-polymerase chain reaction (RT-PCR). A PCR product amplified out of reverse-transcribed Mv1Lu mRNA was used to screen a Mv1Lu cDNA library. This yielded one single positive clone that encoded the sequences obtained from the purified protein (Figure 9A). Screening of cDNA libraries from human kidney and mouse embryo with the Kip1 cDNA yielded clones of highly related sequence. The human and mouse Kip1 cDNAs had open reading frames of 594 and 591 bp, respectively, starting with an ATG codon in a favorable translation initiation context and preceded by stop codons (data not shown). Compared to these open reading frames, the mink clone was incomplete, and ended at nucleotide 534 (Figure 9A).

The Kip1 cDNA encodes a predicted protein of 198 amino acids (22,257 daltons) in human and 197 amino acids (22,208 daltons) in mouse. These values are smaller than the 27 kd value obtained with the purified mink protein by SDS-PAGE. To resolve this discrepancy, a cDNA encoding the mouse Kip1 sequence was constructed, and tagged at the C-terminus with a hexahistidine sequence (~1 kd mass). In vitro transcription and translation of this cDNA yielded a product that bound specifically to Ni⁺⁺-NTA-agarose and migrated as a 28 kd protein on SDS-PAGE gels (Figure 8C), confirming that the cloned cDNA encodes full-length Kip1 and that this protein migrates on SDS-PAGE somewhat slower than its calculated molecular mass.

Kip1 is highly conserved and related to Cip1/WAF1

The predicted human, mouse and mink Kip1 amino acid sequences are highly related, showing ~90 % identity (Figure

9A). A Genbank search revealed that, at the amino acid level, Kip1 shows significant homology only to Cip1/WAF1. The similarity was largely limited to a 60-amino acid segment in the N-terminal half of the protein. This region
5 was 44% identical to the corresponding region in Cip1/WAF1 (Figure 9B). Like Cip1/WAF1, Kip1 has a putative bipartite nuclear localization signal (Dingwall and Laskey, 1991) near the C-terminus (Figure 9B). Yet unlike Cip1/WAF1, the Kip1
10 sequence does not have a putative zinc finger motif in the N-terminal region, and has a C-terminal extension of 23 amino acids that contains a consensus Cdc2 phosphorylation site (Figure 9B).

Cdk inhibitory activity

15 Pure recombinant Kip1 tagged with hexahistidine at the C-terminus inhibited the histone H1 kinase activity of human cyclin A-Cdk2, cyclin E-Cdk2 and cyclin B1-Cdk2 complexes when assayed under linear reaction conditions (Figures 10A and 10B) whereas a mock sample from bacteria transformed
20 with vector alone did not. Cyclin E-Cdk2 was inhibited half-maximally at 0.5 nM Kip1 (Figure 10B). Complete inhibition of cyclin A-Cdk2 required an eight-fold higher concentration, and this concentration was not sufficient to completely block cyclin B1-Cdk2 (Figure 10B). Addition of
25 Kip1 to cyclin E-Cdk2, cyclin A-Cdk2 or cyclin D2-Cdk4 complexes inhibited their ability to phosphorylate a GST-Rb fusion product (Figure 10C and D). The relative sensitivity of cyclin E-Cdk2 and cyclin A-Cdk2 to inhibition by Kip1 in these assays paralleled their sensitivity in the histone H1
30 kinase assays (compare Figures 10B and 10D). Approximately 10 nM cyclin and 10 nM Cdk were used in these assays, but the actual concentration of cyclin:Cdk complexes is not known.

35 Cdk inhibitory domain

It was investigated whether the inhibitory activity of Kip1 resided in the region of similarity to Cip1/WAF1. A 52-amino acid peptide [Kip1(28-79)] corresponding to this region in Kip1 (Figure 10E) was produced recombinantly and purified with a C-terminal hexahistidine tag. This peptide inhibited Rb phosphorylation by cyclin A-Cdk2 with a potency that was close to that of full length Kip1 (Figure 10E) and inhibited cyclin E-Cdk2 or cyclin D2:Cdk4 less effectively. Versions of this Kip1 region missing three amino acids at the N-terminus or fifteen at the C-terminus, were much weaker as Cdk inhibitors, and deletion of seven N-terminal amino acids yielded a product with no inhibitory activity (Figure 10E). The peptide Kip1[(104-152)] which has little sequence similarity to Cip1/WAF1, was inactive as a Cdk inhibitor (Figure 10E).

Kip1 prevents Cdk2 activation

Kip1 was originally identified as a factor whose presence in extracts from quiescent cells rendered Cdk2 refractory to activation by phosphorylation at Thr¹⁶⁰. In order to determine if Kip1 could block Cdk activation, its effect on cyclin E-dependent Cdk2 activation in extracts from exponentially growing cells was assayed (Koff et al., 1993). A549 human lung carcinoma cell extracts were incubated with histidine-tagged cyclin E which was then retrieved and assayed for associated histone H1 kinase activity (Figure 11A). Addition of histidine-tagged Kip1 to the cell extracts markedly decreased the level of cyclin E-associated kinase activity (Figure 11A). In parallel assays, the retrieved cyclin E was subjected to SDS-PAGE and western blotting with anti-Cdk2 antibodies. Cell extracts that did not receive Kip1 yielded cyclin E-associated Cdk2 in a form that corresponds to Cdk2 phosphorylated at Thr¹⁶⁰ (Gu et al., 1992) (Figure 11B). In contrast, cyclin E-associated Cdk2

from extracts that received Kip1 was exclusively in the inactive form (Figure 11B). Collectively, these results suggested that Kip1 binding to preactive cyclin E-Cdk2 complexes in vitro prevented Thr¹⁶⁰ phosphorylation and activation of Cdk2.

Kip 1 overexpression inhibits cell entry into S phase

Mouse Kip1 subcloned into a mammalian expression vector was transfected into Mv1Lu cells under conditions in which up to 65% of the cell population takes up and transiently expresses transfected plasmids (Attisano et al., 1993). The rate of ¹²⁵I-deoxyuridine incorporation into DNA was reduced 70% in cells transfected with Kip1 compared to cells transfected with vector alone (Table III).

Table III

Kip1 blocks entry into S phase

Time after transfection	Vector	¹²⁵ I-deoxyuridine incorporation _a	Percentage of CD16 ⁺ cells in S phase ^b
24 h	pCMV5	27,581 ± 5,126	26 ± 5
	pCMV5-Kip1	8,386 ± 1,250	9 ± 2
43 h	pCMV5	5,126 ± 47	35 ± 2
	pCMV5-Kip1	1,510 ± 140	7 ± 1

The TGF- β receptor-defective R-1B cell line was cotransfected with a human CD16 expression vector and pCMV5 or pCMV5 containing the mouse Kip1 cDNA. Assays were conducted at the indicated times after transfection. a, ¹²⁵I-deoxyuridine incorporated over a 3 h period by the entire cell population. Data are the average ± S.D. of triplicate determinations. b, Transfected cells were immunostained with anti-CD16 and analyzed for DNA content. Data are the average of two separate experiments, and show the range of values.

To determine the effect on cell cycle distribution, Kip1 was cotransfected with a CD16 expression vector (Kurosaki and Ravetch, 1989) that allowed flow cytometric separation of the transfected cells based on CD16 immunofluorescence. The CD16⁺ population cotransfected with Kip1 showed a larger proportion of cells in G1 phase and a smaller proportion in S phase than the CD16⁺ population cotransfected with vector alone (Table III), suggesting that Kip1 overexpression obstructed cell entry into S phase. Cell numbers after transfection indicated that Kip1 did not cause cell death (data not shown).

Kip1 mRNA distribution and levels in quiescent and proliferating cells

The level of endogenous Kip1 mRNA expression in various human tissues was determined by Northern blot analysis. The only mRNA detected was a species of 2.5 kb present at similar levels in all tissues tested, although it was somewhat higher in skeletal muscle and lower in liver and kidney (Figure 12A). Kip1 mRNA levels were similar in exponentially proliferating and contact-inhibited Mv1Lu cells, and did not change when cells were released from contact inhibition by being plated at low density in the presence of serum (Figure 12B). Addition of TGF- β to cells released from contact-inhibition also did not affect Kip1 mRNA levels (Figure 12B). These results indicate that the regulation of Kip1 by extracellular antiproliferative signals occurs at a post-transcriptional level.

30

Discussion

A family of Cdk inhibitors

Human Kip1 encodes a protein of 198 amino acids that is

highly conserved (~90 % identity) in mouse and mink. Its most distinctive feature is a 60-amino acid region in the N-terminal half that has amino acid sequence similarity to Cip1/WAF1 (El-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993). Like Cip1/WAF1, Kip1 contains a potential nuclear localization signal in the C-terminal region. In Kip1, this region also contains a consensus Cdc2 kinase site that might play a role in feed-back regulation by their target kinases.

10

The structural similarity between Kip1 and Cip1/WAF1 defines a family of mammalian Cdk inhibitors with different regulatory properties. Kip1 is involved post-transcriptionally in the action of extracellular signals (present work) and its silencing in exponentially growing cells correlates with binding to a heat-labile component. In contrast, Cip1/WAF1 is regulated transcriptionally by p53, senescence and cell quiescence. Kip1 and Cip1/WAF1 are more effective against G1 Cdks than against mitotic Cdks. However, Kip1 was more effective against cyclin E-Cdk2 than against cyclin A-Cdk2 (or cyclin D2-Cdk4) whereas in similar assays, Cip1/WAF1 was more effective against cyclin A-Cdk2 (Harper et al., 1993). Kip1 effectiveness is likely defined by its binding affinity for a given cyclin-Cdk complex.

25

The Kip1 region that is similar to Cip1/WAF1 is sufficient to inhibit Cdk activity when tested as a 52-amino acid peptide in vitro. This 52 amino acid segment contains the sequence LFGPVN which corresponds to the longest uninterrupted stretch of identity to Cip1/WAF1 and, interestingly, is similar to the FAR1 sequence LSQPVN located in a region required for interaction with CLN2-CDC28 (Peter et al., 1993).

35 Cdk inhibition at two levels

Kip1 can inhibit both the process of Cdk activation and the kinase activity of cyclin-Cdk complexes assembled and activated in intact cells. Kip1 was originally identified as a factor whose presence in extracts of quiescent cells rendered them unable to activate Cdk2 by phosphorylation at Thr¹⁶⁰. Indeed, recombinant Kip1 inhibits Cdk2 Thr¹⁶⁰ phosphorylation and activation in vitro. Although Kip1 could act as an inhibitor of the Cdk-activating kinase, previous results tend to argue against this possibility. The dual effects of Kip1, both on Cdk2 activation and Cdk2 activity, might relate to the fact that Thr¹⁶⁰ is located in a loop that closes the substrate-binding cleft in the Cdk2 structure (DeBondt et al., 1993). It is conceivable that binding of Kip1 to this region might interfere with Thr¹⁶⁰ phosphorylation as well as with the catalytic function of activated Cdk2.

Function in the cell cycle

Cyclin E-Cdk2 and cyclin D-Cdk4 are rate limiting for G1 progression (Jiang et al., 1993; Ohtsubo and Roberts, 1993; Quelle et al., 1993). Inhibition of these kinases by Kip1 in vivo would render cells unable to reach that transition. The strong reductions in the rate of DNA synthesis and the proportion of cells in S phase caused by Kip1 transfection are consistent with this possibility and with a role of Kip1 as mediator of extracellular growth inhibitory signals.

As cells released from contact inhibition move closer to S phase, their extracts contain progressively lower levels of Kip1 activity, and this decline can be prevented by TGF- β addition early in G1 phase. However, the present results show that contact-inhibited cells and TGF- β -treated cells have Kip1 mRNA levels equal to those of proliferating cells. Furthermore, extracts from proliferating cells yield active

Kip1 when they are heated transiently at 100°C. One interpretation of these observations is that Kip1 is progressively sequestered by binding to a heat-labile component as cells progress through G1, and this process can
5 be prevented by TGF- β . Mitogens and antimitogens might regulate Kip1 activity or availability by controlling its binding to a silencing protein. Alternatively, Kip1 might be a passive regulator whose uniform levels could ensure that active Cdks become available only when their levels reach
10 the threshold imposed by binding to Kip1. In the latter situation, even small effects of mitogens and antimitogens on cyclin or Cdk protein levels could become amplified by the existence of that threshold.

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15

What is claimed is:

1. An isolated protein having an apparent molecular weight of about 27 kD as measured by SDS polyacrylamide gel electrophoresis, and capable of binding to and inhibiting the activation of a cyclin E-Cdk2 complex.
5
2. A recombinant nucleic acid molecule which encodes the protein of claim 1.
10
3. The recombinant nucleic acid molecule of claim 2, wherein the nucleic acid molecule is a DNA molecule.
4. The recombinant nucleic acid molecule of claim 3, wherein the DNA molecule is a cDNA molecule.
15
5. The recombinant nucleic acid molecule of claim 2, wherein the nucleic acid molecule is an RNA molecule.
- 20 6. A vector comprising the recombinant nucleic acid molecule of claim 4.
7. The vector of claim 6, wherein the vector is a plasmid.
- 25 8. The vector of claim 6, wherein the vector is a virus.
9. A host vector system for the production of a protein having an apparent molecular weight of about 27 kD as measured by SDS polyacrylamide gel electrophoresis, and capable of binding to and inhibiting the activation of a cyclin E-Cdk2 complex, which comprises the vector of claim 6 in a suitable host.
30
10. The host vector system of claim 9, wherein the suitable host is a bacterial cell.
35

11. The host vector system of claim 9, wherein the suitable host is an eucaryotic cell.
12. The host vector system of claim 11, wherein the eucaryotic cell is an insect cell.
13. A method for producing a protein having an apparent molecular weight of about 27 kD as measured by SDS polyacrylamide gel electrophoresis, and capable of binding to and inhibiting the activation of a cyclin E-Cdk2 complex, which comprises growing the host vector system of claim 9 under conditions permitting the production of the protein and recovering the protein produced thereby.
14. A method of determining whether an agent is capable of specifically inhibiting the ability of p27 protein to inhibit the activation of cyclin E-Cdk2 complex which comprises:
 - (a) contacting suitable amounts of p27 protein, cyclin E, Cdk2 and the agent under suitable conditions;
 - (b) subjecting the p27, cyclin E, Cdk2, and agent so contacted to conditions which would permit the formation of active cyclin E-Cdk2 complex in the absence of p27 protein;
 - (c) quantitatively determining the amount of active cyclin E-Cdk2 complex so formed; and
 - (d) comparing the amount of active cyclin E-Cdk2 complex so formed with the amount of active cyclin E-Cdk2 complex formed in the absence of the agent, a greater amount of active cyclin E-Cdk2 complex formed in the presence of the agent than in the absence of the agent indicating that the agent is capable of specifically inhibiting

the ability of p27 protein to inhibit the activation of cyclin E-Cdk2 complex.

15. A method of determining whether an agent is capable of specifically enhancing the ability of p27 protein to inhibit the activation of cyclin E-Cdk2 complex which comprises:
- (a) contacting suitable amounts of p27 protein, cyclin E, Cdk2 and the agent under suitable conditions;
 - (b) subjecting the p27, cyclin E, Cdk2, and agent so contacted to conditions which would permit the formation of active cyclin E-Cdk2 complex in the absence of p27 protein;
 - (c) quantitatively determining the amount of active cyclin E-Cdk2 complex so formed; and
 - (d) comparing the amount of active cyclin E-Cdk2 complex so formed with the amount of active cyclin E-Cdk2 complex formed in the absence of the agent, a lesser amount of active cyclin E-Cdk2 complex formed in the presence of the agent than in the absence of the agent indicating that the agent is capable of specifically enhancing the ability of p27 protein to inhibit the activation of cyclin E-Cdk2 complex.
16. A method of treating a subject having a hyperproliferative disorder which comprises administering to the subject a therapeutically effective amount of an agent capable of specifically enhancing the ability of p27 protein to inhibit the activation of cyclin E-Cdk2 complex in the hyperproliferative cells of the subject, so as to thereby treat the subject.
17. The method of claim 16, wherein the subject is a human.

18. The method of claim 16, wherein the hyperproliferative disorder is selected from the group consisting of cancer and hyperplasia.
- 5 19. A method of treating a subject having a hypoproliferative disorder which comprises administering to the subject a therapeutically effective amount of an agent capable of specifically inhibiting the ability of p27 protein to inhibit the activation of cyclin E-Cdk2
10 complex in the hypoproliferative cells of the subject, so as to thereby treat the subject.
20. The method of claim 19, wherein the subject is a human.
- 15 21. The method of claim 19, wherein the hypoproliferative disorder is an ulcer.
22. A method of diagnosing a hyperproliferative disorder in a subject which disorder is associated with the
20 presence of a p27 protein mutation in the cells of the subject, which comprises determining the presence of a p27 protein mutation in the cells of the subject, said mutation being associated with a hyperproliferative disorder, so as to thereby diagnose a
25 hyperproliferative disorder in the subject.
23. The method of claim 22, wherein the subject is a human.
24. The method of claim 22, wherein the hyperproliferative
30 disorder is cancer.
25. A pharmaceutical composition which comprises an effective amount of a recombinant virus capable of infecting a suitable host cell, said recombinant virus
35 comprising the nucleic acid molecule of claim 2, and a

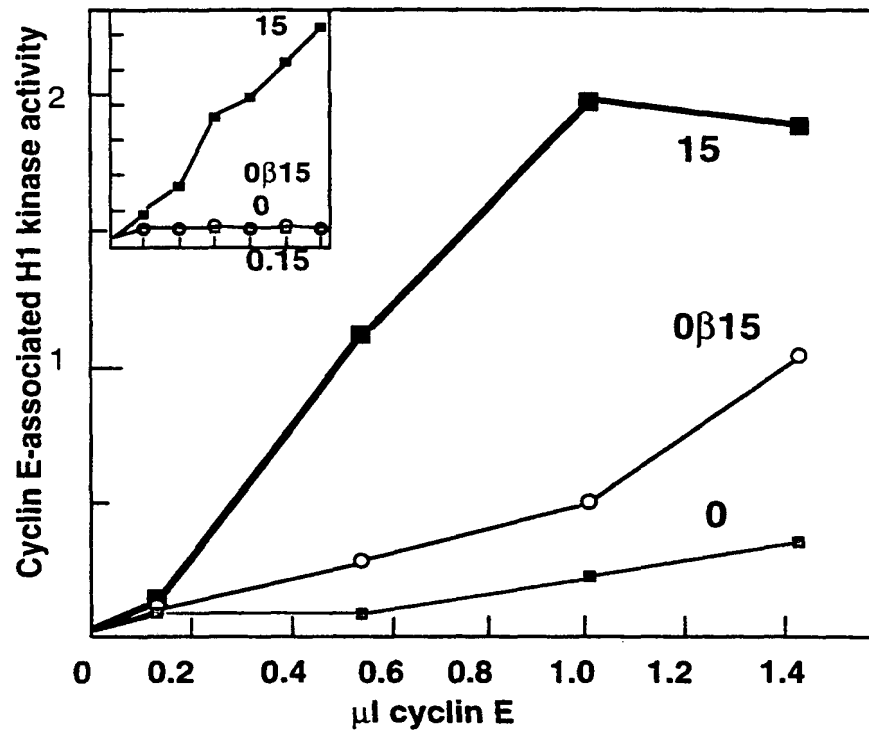
pharmaceutically acceptable carrier.

26. A method for treating a subject suffering from a hyperproliferative disorder associated with the presence of a p27 protein mutation in the cells of the subject, which comprises administering to the subject an amount of the pharmaceutical composition of claim 25 effective to treat the subject.
27. The method of claim 26, wherein the subject is a human.
28. The method of claim 26, wherein the hyperproliferative disorder is cancer.

15

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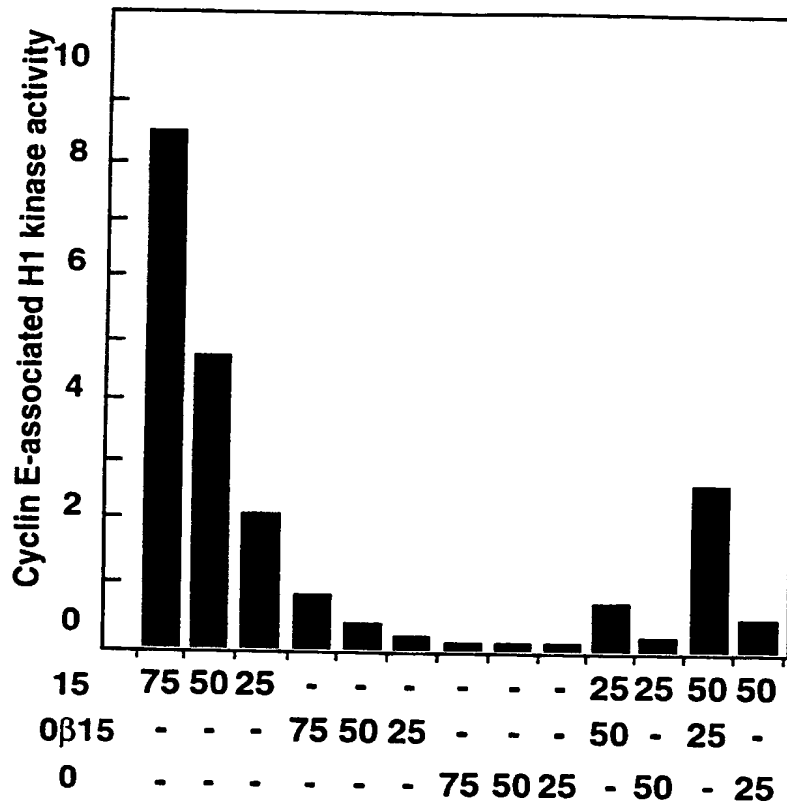
FIGURE 1A



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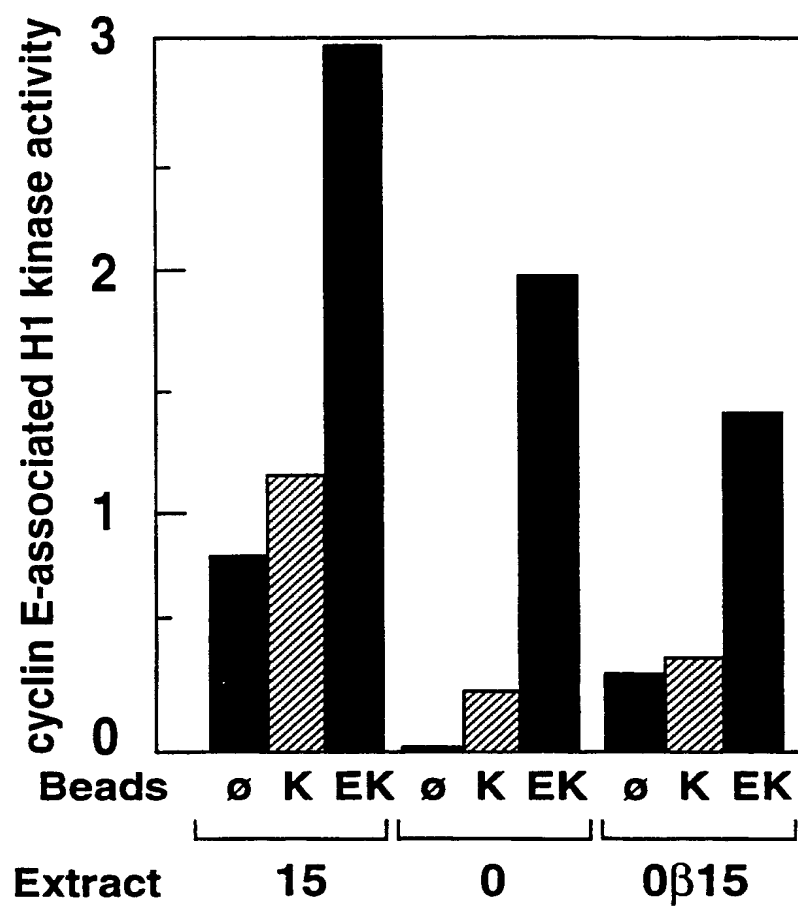
FIGURE 1B



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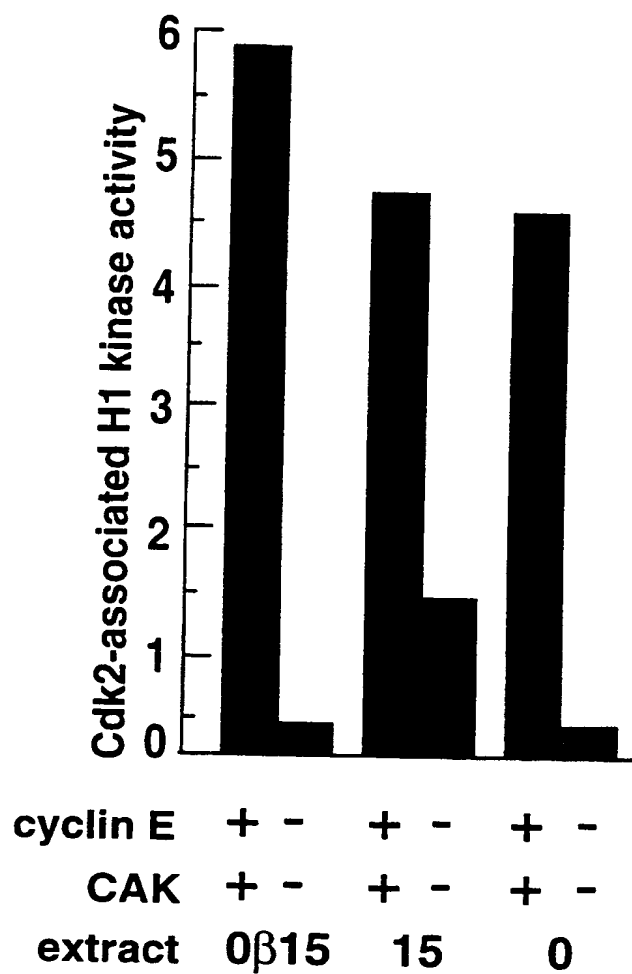
FIGURE 2A



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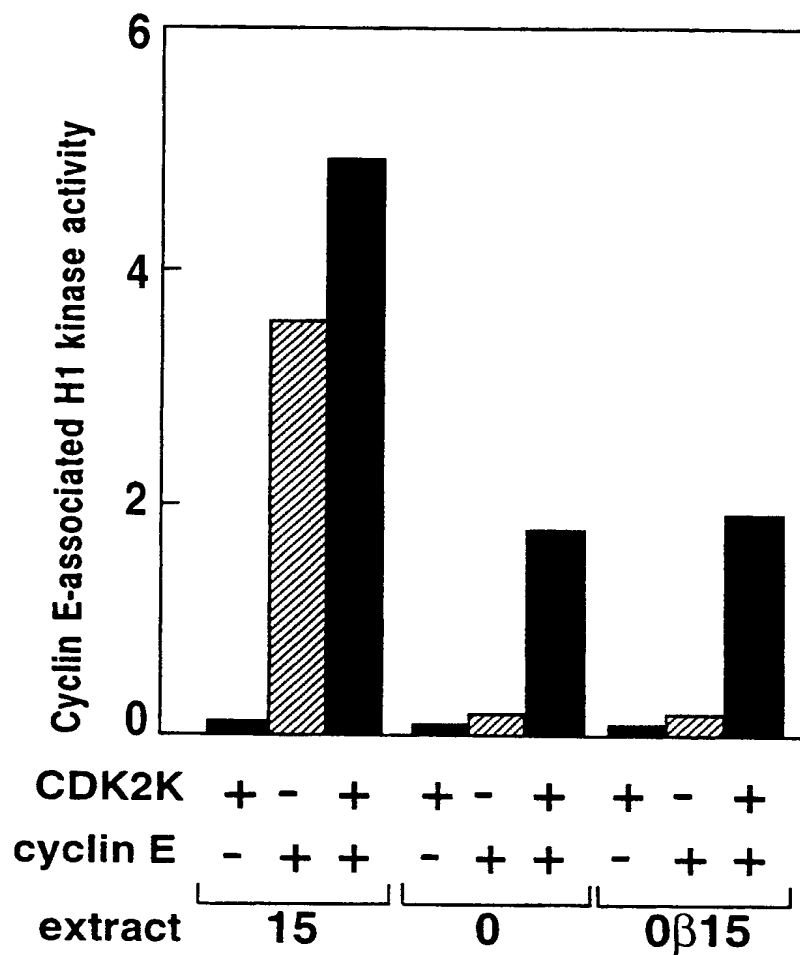
FIGURE 2B



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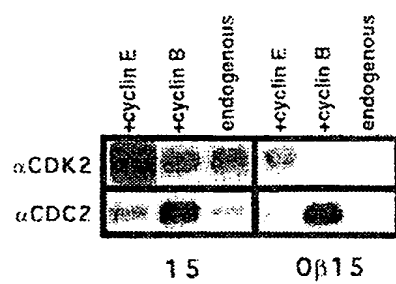
FIGURE 2C



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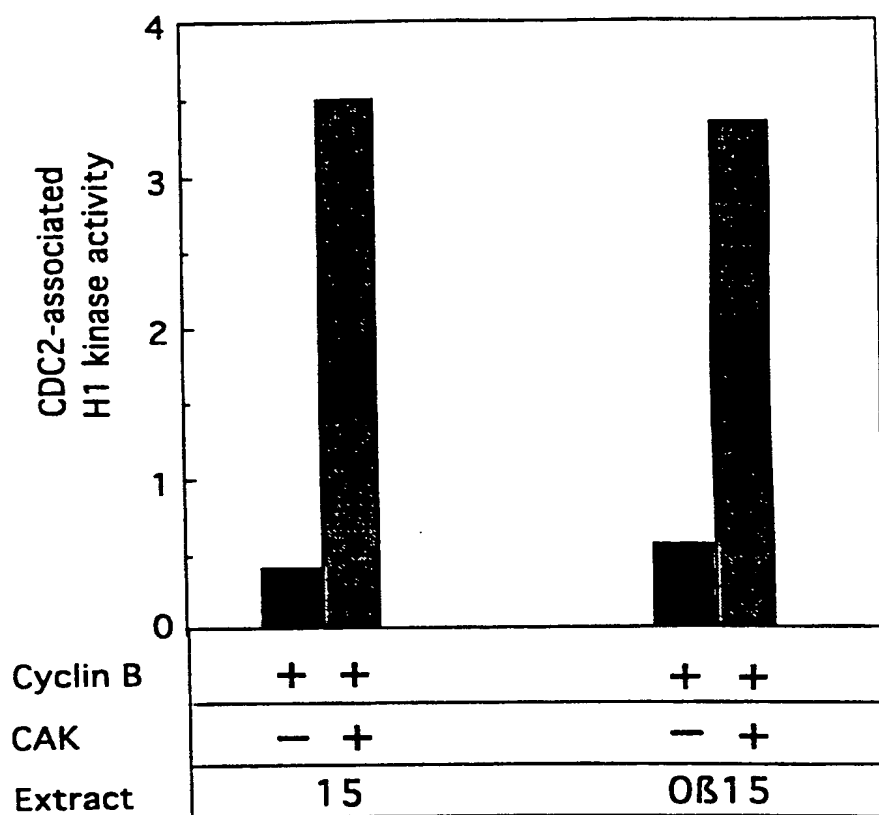
FIGURE 3A



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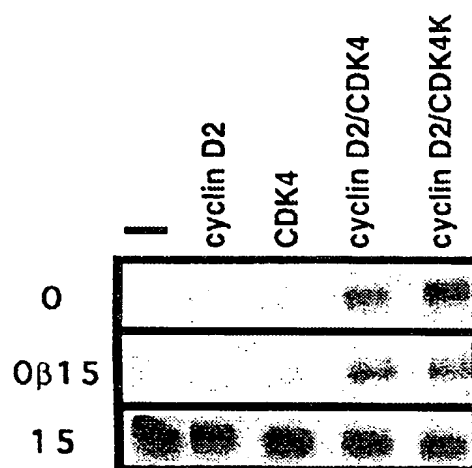
FIGURE 3B



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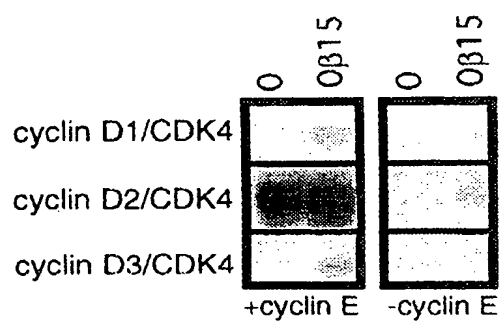
FIGURE 4A



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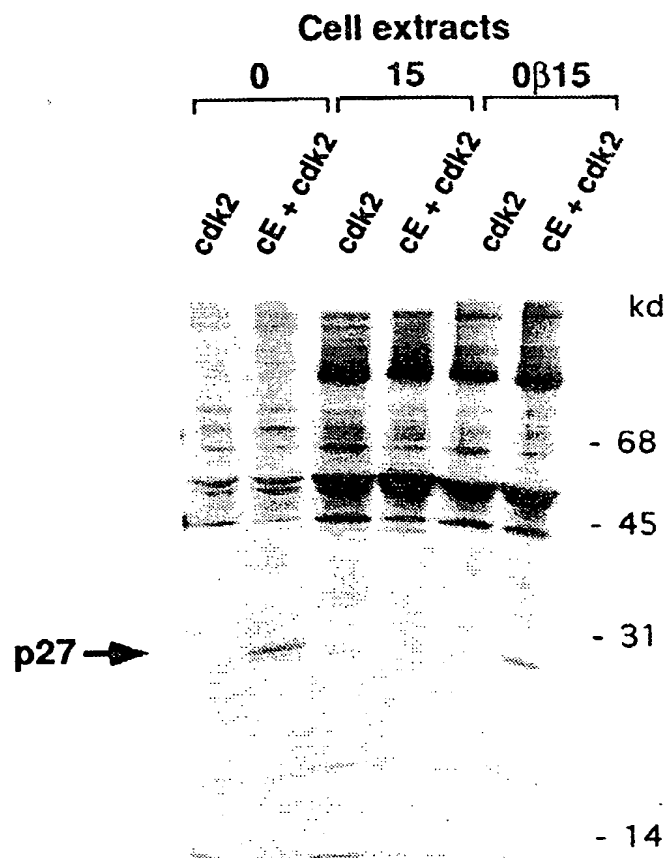
FIGURE 4B



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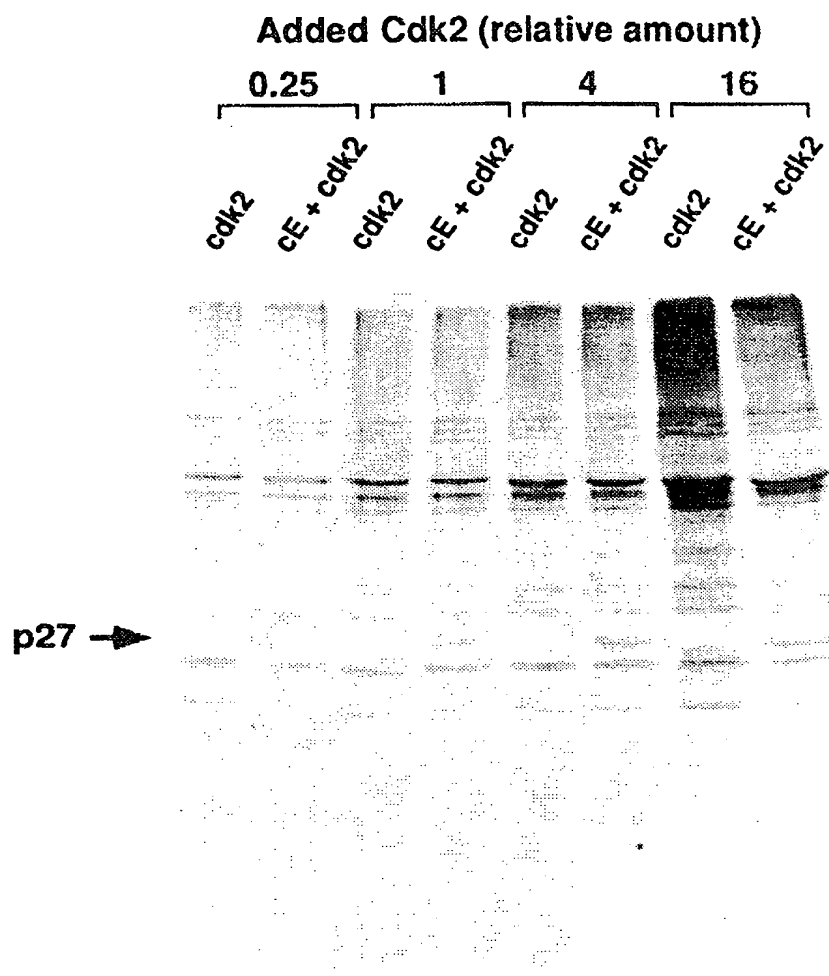
FIGURE 5A



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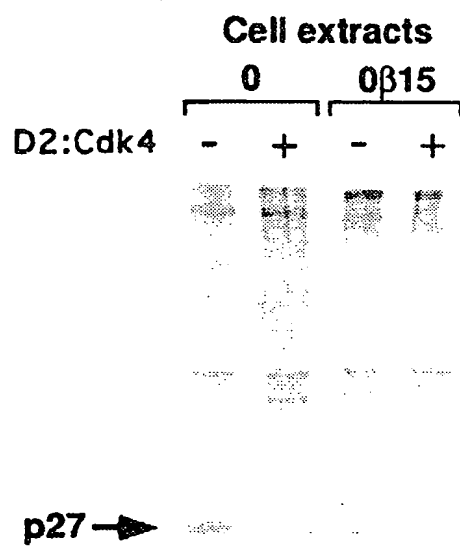
FIGURE 5B



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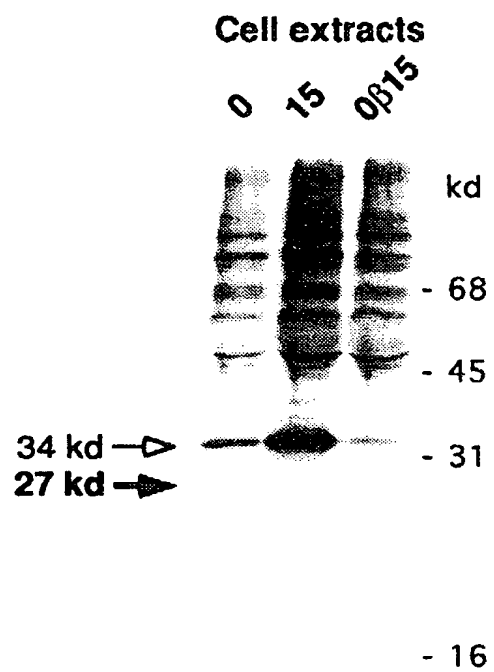
FIGURE 5C



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FIGURE 5D

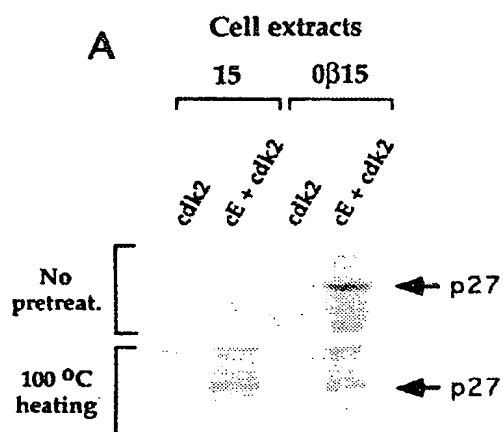


anti-Cdk4 i.p.

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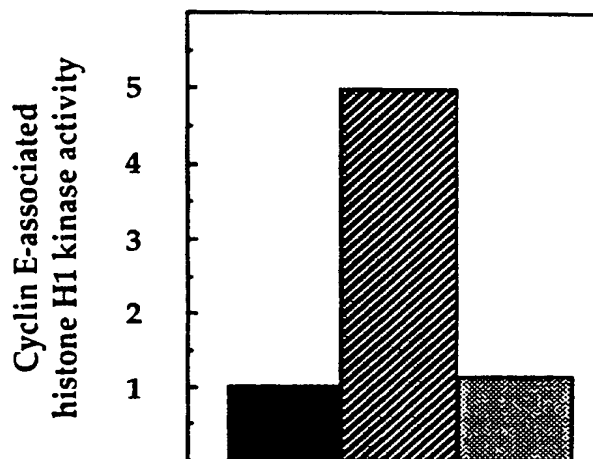
FIGURE 6A



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FIGURE 6B



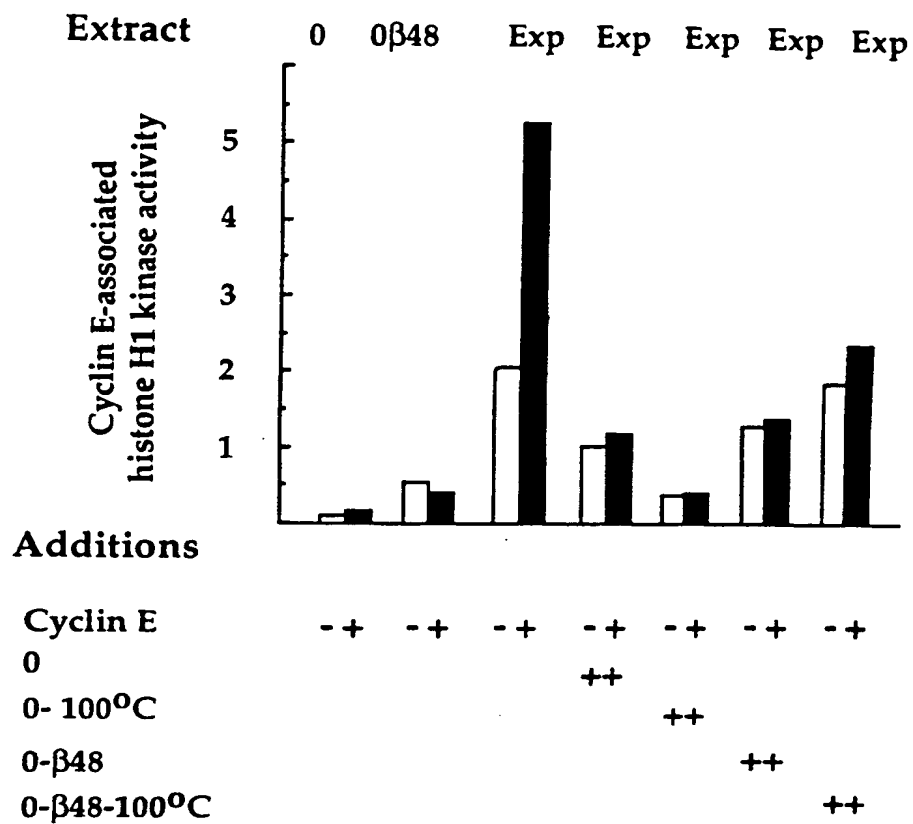
Additions:

100°C-Heated Exp. cell extract	-	-	+
Cyclin E	-	+	+

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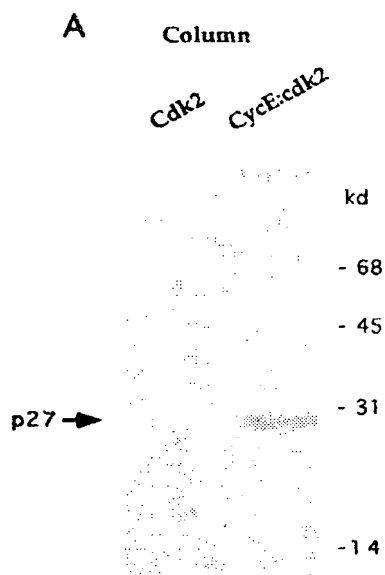
FIGURE 6C



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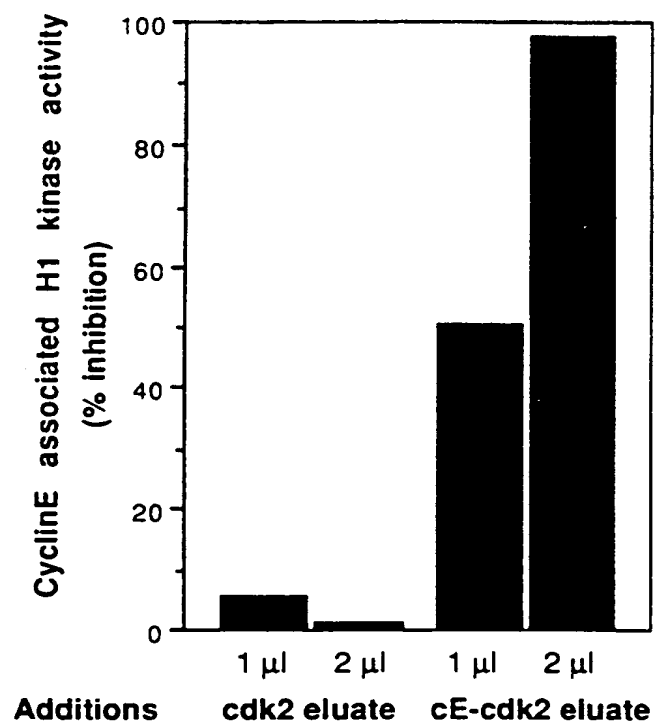
FIGURE 7A



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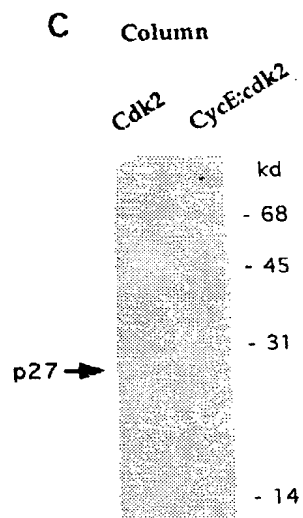
FIGURE 7B



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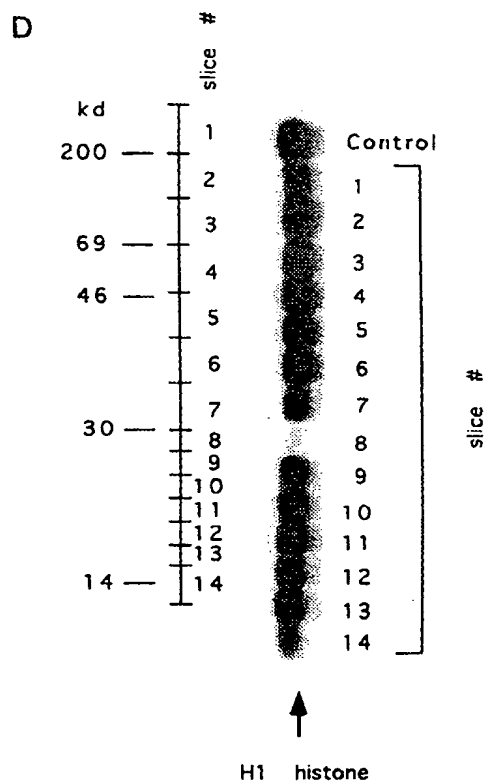
FIGURE 7C



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FIGURE 7D



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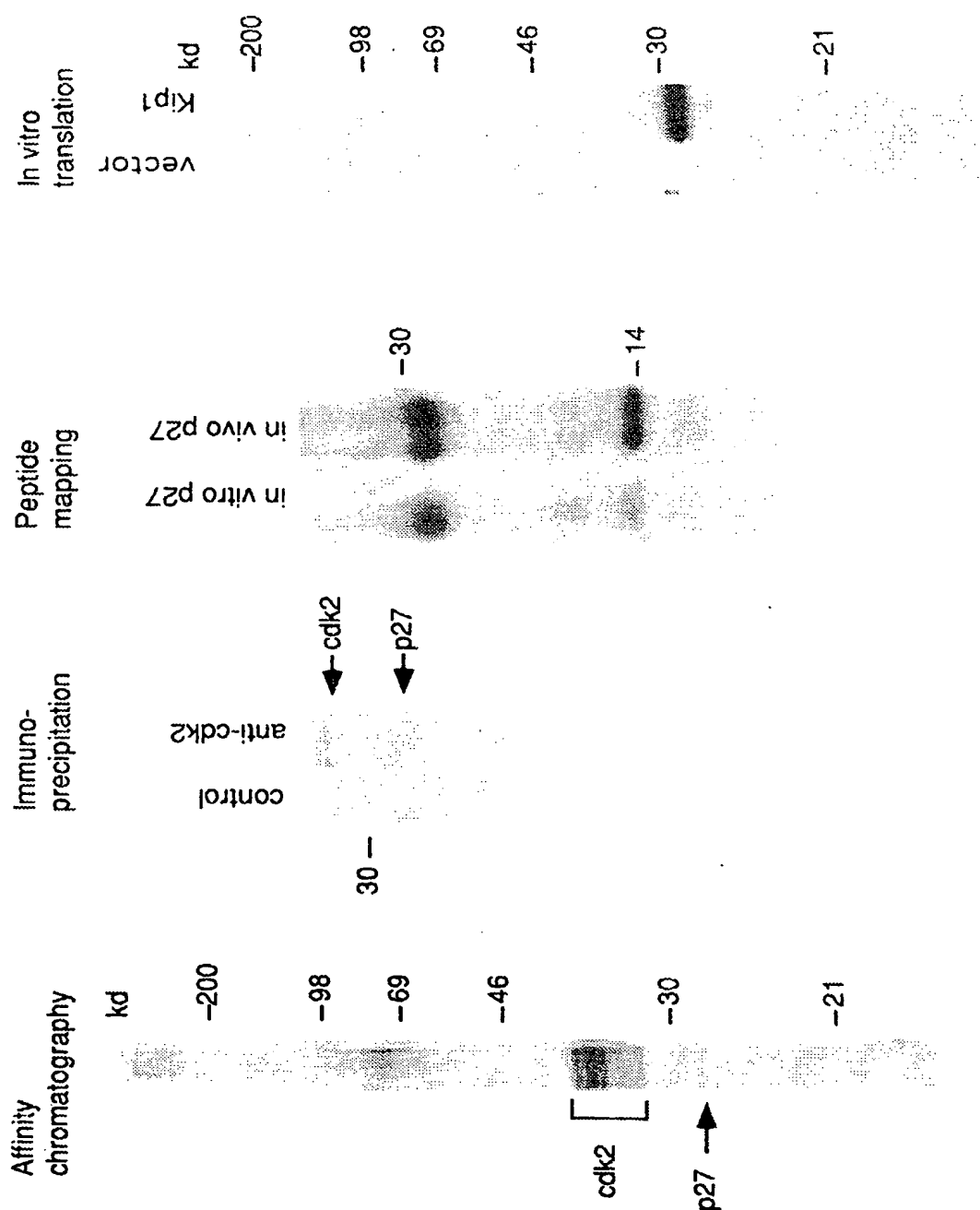
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FIGURE 8A

FIGURE 8B

FIGURE 8C

FIGURE 8D



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FIGURE 9A

		20	40
mk	kipl	MSNVRVSNNGSPSLERMDARQAEYPKPSACRN <u>LF</u> GPVNHEELTRDLEKHRR	
m	kiplDH.....C.	
h	kiplH.....D.....C.	
		70	90
mk	kipl	DMEEASQRKWNFDQNHKPLEGKYEWQEVKGS <u>LP</u> EFYRPPRPPKGACK	
m	kiplR.....R.....S...	
h	kipl	
		120	140
mk	kipl	VPAQESQDVSGTRQAVPLMG <u>SQ</u> ANSEDTLVDQKTD <u>TAD</u> NOAGLAECCTG	
m	kipl	.L.....S.....I.....R.....MP.SS.....P.	
h	kiplS.P.A..I.AP.....P...PS.S.T...DR.A.	
		170	190
mk	kipl	IRKRPATDDSSPQNKPRANRTEENVSDGSxxxxxxxxxxxxxxxxxxxx	
m	kipl	M.....AE...S.....PNAGTVEQTPKKPGLRR-QT	
h	kiplT.....S.....R..	

FIGURE 9B

h	kipl	MSNVRVSNNGSPSLERMDARQAEHPKPSACRN <u>LF</u> GPVHHEELTRDLEKHCR	50
h	cipl	MSEPAGDVQRNPGSGHACR <u>LF</u> GPVHSEQLSRDCDALMA	39
h	kipl	DMEEASQRKWNFDQNHKPLEGKYEWQEVKGS <u>LP</u> EFYRPPRPPKGACK	100
h	cipl	GCTQEAREFWNFDEVTEPLEGDFWEPVRLGLLEKLMPTGPRGRDEL	89
h	kipl	VPAQESQDVSGSRPAAPLIGAPANSEDTLVDPKTDPSDSQTGLADRCAG	150
h	cipl	GGGRRPGTSPALLQGTAEEDHVDLSLSCTLVPRSGEQAEAGSFGGPGDSOG	139
h	kipl	IRKRPATDDSSSTQ-NKPRANRTEENVSDGSPNAGSVECTPKKPGLRRRQT*	198
h	cipl	IRKPRCTSMTDFYHSHKRLIFSRRKP*	164

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FIGURE 10C

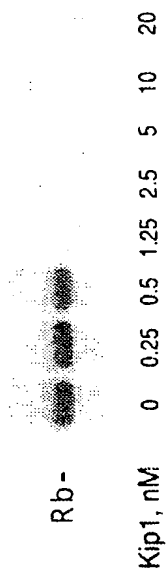


FIGURE 10A

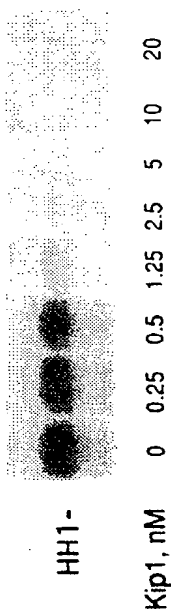


FIGURE 10D

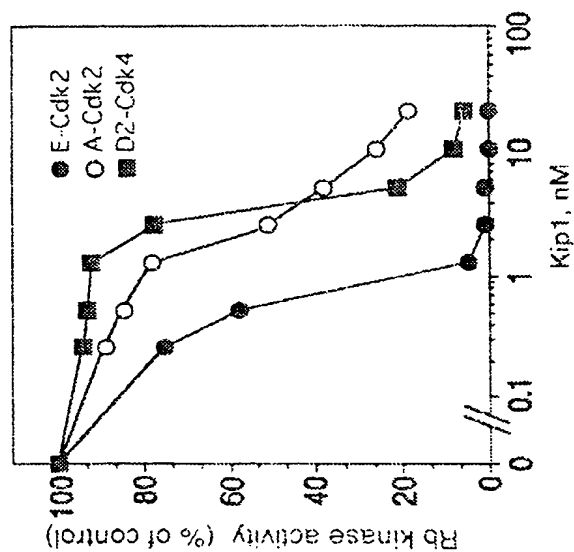
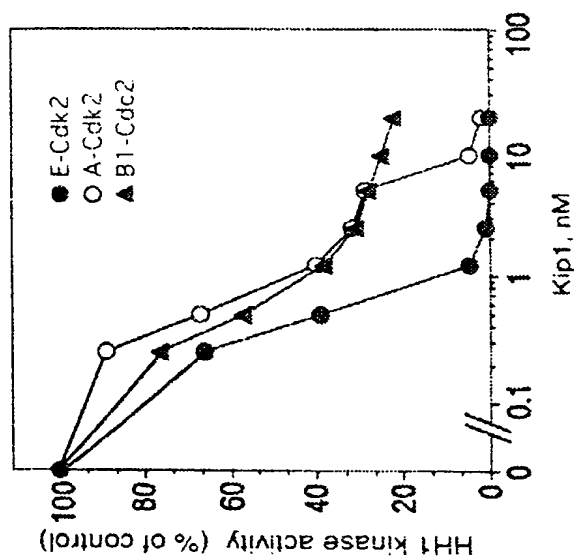


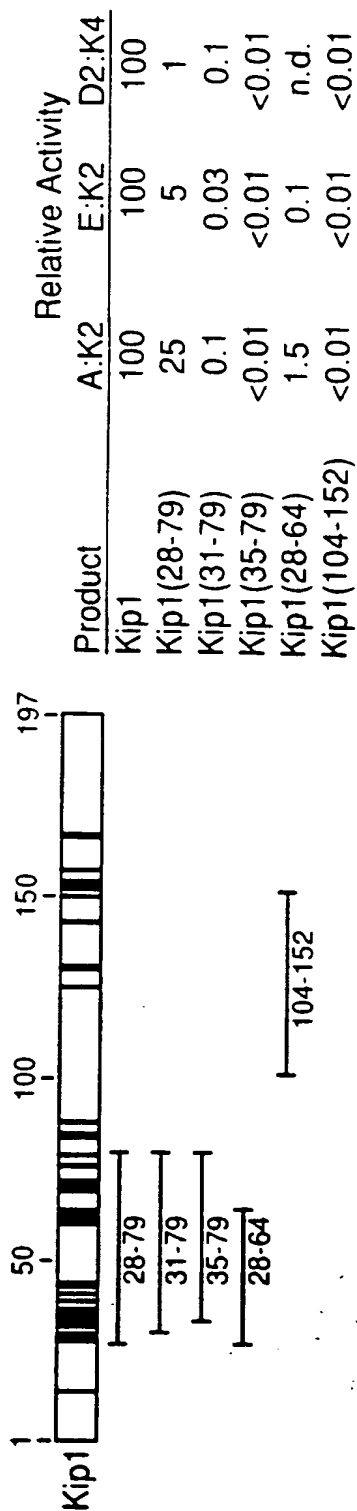
FIGURE 10B



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FIGURE 10E



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FIGURE 11A

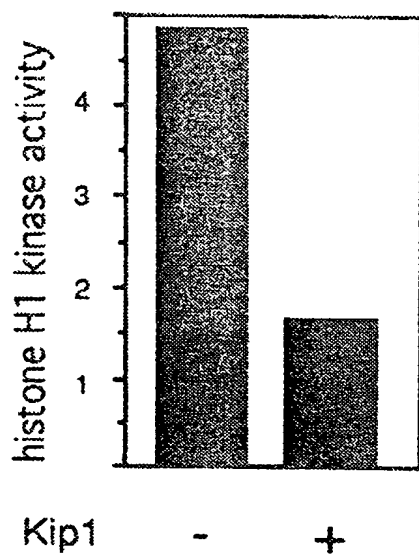
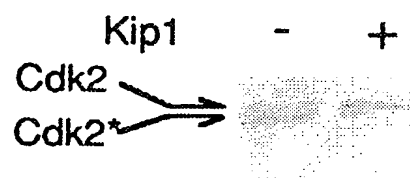


FIGURE 11B



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FIGURE 12A

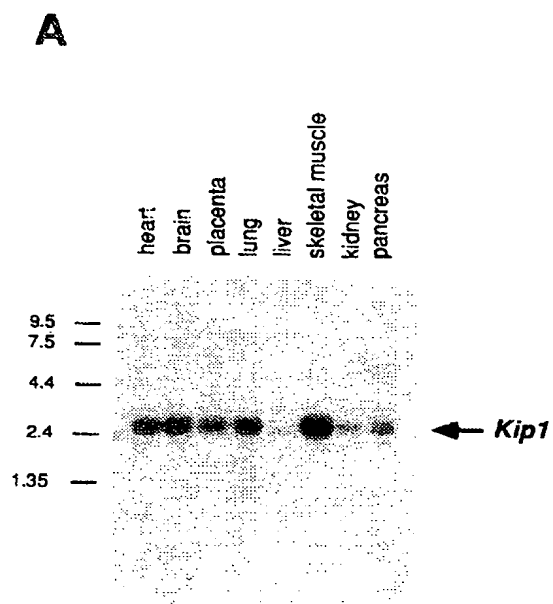
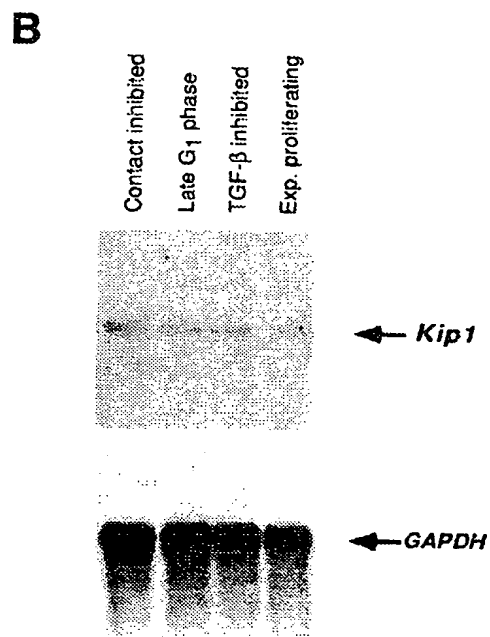


FIGURE 12B



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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/00247

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : 435/7.1, 69.1, 240.2, 252.3, 320.1; 514/2; 530/350; 536/23.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 69.1, 240.2, 252.3, 320.1; 514/2; 530/350; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, MEDLINE, WPI

search terms: cyclin E-Cdk2 inhibitor, binding to cyclin E-Cdk2, p27

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	Cell, Volume 75, issued 19 November 1993, Harper et al, "The p21 Cdk-Interacting Protein Cip1 is a Potent Inhibitor of G1 Cyclin-Dependent Kinases", pages 805-816, see pages 806-811.	1-13, 25 ----- 14-24, 26-28
Y	Science, Volume 257, issued 18 September 1992, Koff et al, "Formation and Activation of a Cyclin E-cdk2 Complex During the G ₁ Phase of the Human Cell Cycle", pages 1689-1694, see pages 1690-1693.	1-28
P, X ----- P, Y	Cell, Volume 78, issued 15 July 1994, Polyak et al, "Cloning of p27 ^{kip1} , a Cyclin-Dependent Kinase Inhibitor and a Potential mediator of Extracellular Antimitogenic Signals", pages 59-66, see pages 59-63.	1-13, 25 ----- 14-24, 26-28

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

04 APRIL 1995

Date of mailing of the international search report

20 APR 1995

 Name and mailing address of the ISA/US
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/00247

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X ----- P, Y	Genes & Development, Volume 8, issued 27 January 1994, Polyak et al, "p27 ^{Kip1} , a Cyclin-Cdk Inhibitor, Links Transforming Growth Factor- β and Contact Inhibition to Cell Cycle Arrest", pages 9-22, see pages 10-15.	1, 25 ----- 2-24, 26-28

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/00247**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)★

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07K 14/435; C12N 5/06, 15/70, 15/74, 15/79; C07H 21/00; A61K 38/17

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claims 1-13 and 25, drawn to the p27 protein and its encoding DNA.

Group II, claims 14 and 15, drawn to a method of determining whether an agent is capable of altering the ability of P27 to inhibit the activation of cyclin E-Cdk2 complex.

Group III, claims 16-21 and 26-28, drawn to a method of treatment.

Group IV, claims 22-24, drawn to a method of diagnosis.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I is drawn to products. Groups II-IV are drawn to materially distinct methods with different goals, steps, and reagents for using the products of Group I. Note PCT Rule 13 does not provide for multiple products or methods within a single application.

